(51) International Patent Classification 6 : C12N 5/06, 5/10, 7/01, 7/02, 15/86	A1	(11) International Publication Number: WO 98/17783
		(43) International Publication Date: 30 April 1998 (30.04.98)

(21) International Application Number: PCT/US97/19541

(22) International Filing Date: 23 October 1997 (23.10.97)

(30) Priority Data:
08/735,609   23 October 1996 (23.10.96)   US

(71) Applicant: REGENTS OF THE UNIVERSITY OF MICHI-GAN [US/US]; 3003 South State Street, Ann Arbor, MI 48109-1280 (US).

(72) Inventors: CHAMBERLAIN, Jeffrey, S.; 1209 Henry, Ann Arbor, MI 48104 (US). AMALFITANO, Andrea; 3305 Middle Brook Court, Durham, NC 27705 (US). HAUSER, Michael, A.; 13774 Sanford Road, Milan, MI 48160 (US). KUMAR-SINGH, Rajendra; Apartment 204, 219 S. Barrington Avenue, Los Angeles, CA 90049 (US). HARTIGAN-O'CONNOR, Dennis, J.; 1106 Maiden Lane Court #109, Ann Arbor, MI 48105 (US).

(74) Agents: CARROLL, Peter, G. et al.; Medlen & Carroll, LLP, Suite 2200, 220 Montgomery Street, San Francisco, CA 94104 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

(54) Title: IMPROVED ADENOVIRUS VECTORS

(57) Abstract

The present invention provides improved adenovirus vectors and packaging cell lines. One type of improved adenoviral vector comprises deletions within the E2b region of the adenoviral genome. These E2b-deleted virus are used in conjunction with novel cell lines that constitutively express E2b gene products. The present invention further provides adenoviral vectors deleted for all viral coding regions. These "gutted" vectors permit the transfer of large genes to cells as demonstrated herein by the transfer of the dystrophin gene to the muscle of mice. The E2b-deleted vectors and the gutted vectors provide improved adenoviral vectors useful for a wide variety of gene therapy applications.

IMPROVED ADENOVIRUS VECTORS

FIELD OF THE INVENTION

The invention relates to improved adenovirus vectors. and more specifically.
adenovirus vectors useful for gene therapy.

BACKGROUND

Adenoviruses (Ad) are double-stranded DNA viruses. The genome of adenoviruses
(~36 kb) is complex and contains over 50 open reading frames (ORFs). These ORFs are
overlapping and genes encoding one protein are often embedded within genes coding for other
Ad proteins. Expression of Ad genes is divided into an early and a late phase. Early genes
are those transcribed prior to replication of the genome while late genes are transcribed after
replication. The early genes comprise E1a. E1b. E2a. E2b. E3 and E4. The E1a gene
products are involved in transcriptional regulation: the E1b gene products are involved in the
shut-off of host cell functions and mRNA transport. E2a encodes the a DNA-binding protein
(DBP): E2b encodes the viral DNA polymerase and preterminal protein (pTP). The E3 gene
products are not essential for viral growth in cell culture. The E4 region encodes regulatory
protein involved in transcriptional and post-transcriptional regulation of viral gene expression:
a subset of the E4 proteins are essential for viral growth. The products of the late genes (e.g.,
L1-5) are predominantly components of the virion as well as proteins involved in the
assembly of virions. The VA genes produce VA RNAs which block the host cell from
shutting down viral protein synthesis.

Adenoviruses or Ad vectors have been exploited for the delivery of foreign genes to
cells for a number of reasons including the fact that Ad vectors have been shown to be highly
effective for the transfer of genes into a wide variety of tissues *in vivo* and the fact that Ad
infects both dividing and non-dividing cells: a number of tissues which are targets for gene
therapy comprise largely non-dividing cells.

The current generation of Ad vectors suffer from a number of limitations which
preclude their widespread clinical use including: 1) immune detection and elimination of cells
infected with Ad vectors. 2) a limited carrying capacity (about 8.5 kb) for the insertion of
foreign genes and regulatory elements. and 3) low-level expression of Ad genes in cells
infected with recombinant Ad vectors (generally. the expression of Ad proteins is toxic to
cells).

The latter problem was thought to be solved by using vectors containing deletions in the E1 region of the Ad genome (E1 gene products are required for viral gene expression and replication). However, even with such vectors, low-level expression of Ad genes is observed. It is now thought that most mammalian cells contain E1-like factors which can substitute for the missing Ad E1 proteins and permit expression of Ad genes remaining on the E1 deleted vectors.

What is needed is an approach that overcomes the problem of low level expression of Ad genes. Such an approach needs to ensure that adenovirus vectors are safe and non-immunogenic.

SUMMARY OF THE INVENTION

The present invention contemplates two approaches to improving adenovirus vectors. The first approach generally contemplates a recombinant plasmid, together with a helper adenovirus, in a packaging cell line. The helper adenovirus is rendered safe by utilization of *loxP* sequences. In the second approach, "damaged" adenoviruses are employed. While the "damaged" adenovirus is capable of self-propagation in a packaging cell line, it is not capable of expressing certain genes (*e.g.*, the DNA polymerase gene and/or the adenovirus preterminal protein gene).

In one embodiment of the first approach, the present invention contemplates a recombinant plasmid, comprising in operable combination: a) a plasmid backbone, comprising an origin of replication, an antibiotic resistance gene and a eukaryotic promoter element; b) the left and right inverted terminal repeats (ITRs) of adenovirus, said ITRs each having a 5' and a 3' end and arranged in a tail to tail orientation on said plasmid backbone; c) the adenovirus packaging sequence, said packaging sequence having a 5' and a 3' end and linked to one of said ITRs; and d) a first gene of interest operably linked to said promoter element.

While it is not intended that the present invention be limited by the precise size of the plasmid, it is generally desirable that the recombinant plasmid have a total size of between 27 and 40 kilobase pairs. It is preferred that the total size of the DNA packaged into an EAM derived from these recombinant plasmids is about the length of the wild-type adenovirus genome (~36 kb). It is well known in the art that DNA representing about 105% of the wild-type length may be packaged into a viral particle; thus the EAM derived from recombinant plasmid may contain DNA whose length exceeds by ~105% the size of the wild-type genome.

The size of the recombinant plasmid may be adjusted using reporter genes and genes of interest having various sizes (including the use of different sizes of introns within these genes) as well as through the use of irrelevant or non-coding DNA fragment which act as "stuffer" fragments (e.g., portions of bacteriophage genomes).

In one embodiment of the recombinant plasmid, said 5' end of said packaging sequence is linked to said 3' end of said left ITR. In this embodiment, said first gene of interest is linked to said 3' end of said packaging sequence. It is not intended that the present invention be limited by the nature of the gene of interest; a variety of genes (including both cDNA and genomic forms) are contemplated; any gene having therapeutic value may be inserted into the recombinant plasmids of the present invention. For example, the transfer of the adenosine deaminase (ADA) gene is useful for the treatment of ADA- patients; the transfer of the CFTR gene is useful for the treatment of cystitic fibrosis. A wide variety of diseases are known to be due to a defect in a single gene. The plasmids, vectors and EAMs of the present invention are useful for the transfer of a non-mutated form of a gene which is mutated in a patient thereby resulting in disease. The present invention is illustrated using recombinant plasmids capable of generating encapsidated adenovirus minichromosomes (EAMs) containing the dystrophin cDNA gene (the cDNA form of this gene is preferred due to the large size of this gene); the dystrophin gene is non-functional in muscular dystrophy (MD) patients. However, the present invention is not limited toward the use of the dystrophin gene for treatment of MD; the use of the utrophin (also called the dystrophin related protein) gene is also contemplated for gene therapy for the treatment of MD [Tinsley et al. (1993) Curr. Opin. Genet. Dev. 3:484 and (1992) Nature 360:591]; the utrophin gene protein has been reported to be capable of functionally substituting for the dystrophin gene [Tinsley and Davies (1993) Neuromusc. Disord. 3:539]. As the utrophin gene product is expressed in the muscle of muscular dystrophy patients, no immune response would be directed against the utrophin gene product expressed in cells of a host (including a human) containing the recombinant plasmids, Ad vectors or EAMs of the present invention. While the present invention is illustrated using plasmids containing the dystrophin gene, the plasmids, Ad vectors and EAMs of the present invention have broad application for the transfer of any gene whose gene product is missing or altered in activity in cells.

Embodiments are contemplated wherein the recombinant plasmid further comprises a second gene of interest. In one embodiment, said second gene of interest is linked to said 3' end of said right ITR. In one embodiment, said second gene of interest is a reporter gene. A

variety of reporter genes are contemplated. including but not limited to *E. coli* β-galactosidase gene. the human placental alkaline phosphatase gene. the green fluorescent protein gene and the chloramphenicol acetyltransferase gene.

     As mentioned above. the first approach also involves the use of a helper adenovirus in
5   combination with the recombinant plasmid. In one embodiment. the present invention contemplates a helper adenovirus comprising i) first and a second *loxP* sequences. and ii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end. It is preferred that said first *loxP* sequence is linked to the 5' end of said packaging sequence and said second *loxP* sequence is linked to said 3' end of said packaging sequence. In one
10   embodiment. the helper virus comprises at least one adenovirus gene coding region.

     The present invention contemplates a mammalian cell line containing the above-described recombinant plasmid and the above-described helper virus. It is preferred that said cell line is a 293-derived cell line. Specifically. in one embodiment. the present invention contemplates a mammalian cell line. comprising: a) a recombinant plasmid. comprising. in
15   operable combination: i) a plasmid backbone. comprising an origin of replication. an antibiotic resistance gene and a eukaryotic promoter element. ii) the left and right inverted terminal repeats (ITRs) of adenovirus. said ITRs each having a 5' and a 3' end and arranged in a tail to tail orientation on said plasmid backbone. iii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end and linked to one of said ITRs. and iv) a
20   first gene of interest operably linked to said promoter element: and b) a helper adenovirus comprising i) first and a second *loxP* sequences. and ii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end. As noted previously. said helper can further comprise at least one adenovirus gene coding region.

     Overall. the first approach allows for a method of producing an adenovirus
25   minichromosome. In one embodiment. this method comprises: A) providing a mammalian cell line containing: a) a recombinant plasmid. comprising. in operable combination. i) a plasmid backbone. comprising an origin of replication. an antibiotic resistance gene and a eukaryotic promoter element. ii) the left and right inverted terminal repeats (ITRs) of adenovirus. said ITRs each having a 5' and a 3' end and arranged in a tail to tail orientation
30   on said plasmid backbone. iii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end and linked to one of the ITRs. and iv) a first gene of interest operably linked to said promoter element: and b) a helper adenovirus comprising i) first and a

second *loxP* sequences; ii) at least one adenovirus gene coding region, and iii) the adenovirus packaging sequence, said packaging sequence having a 5' and a 3' end; and

B) growing said cell line under conditions such that said adenovirus gene coding region is expressed and said recombinant plasmid directs the production of at least one adenoviral

5   minichromosome. It is desired that said adenovirus minichromosome is encapsidated.

In one embodiment, the present invention contemplates recovering said encapsidated adenovirus minichromosome and, in turn, purifying said recovered encapsidated adenovirus minichromosome. Thereafter, said purified encapsidated adenovirus minichromosome can be administered to a host (*e.g.*, a mammal). Human therapy is thereby contemplated.

10   It is not intended that the present invention be limited by the nature of the administration of said minichromosomes. All types of administration are contemplated, including direct injection (intra-muscular, intravenous, subcutaneous, etc.), inhalation, etc.

As noted above, the present invention contemplates a second approaches to improving adenovirus vectors. In the second approach, "damaged" adenoviruses are employed. In one

15   embodiment, the present invention contemplates a recombinant adenovirus comprising the adenovirus E2b region having a deletion, said adenovirus capable of self-propagation in a packaging cell line and said E2b region comprising the DNA polymerase gene and the adenovirus preterminal protein gene. In this embodiment, said deletion can be within the adenovirus DNA polymerase gene. Alternatively, said deletion is within the adenovirus

20   preterminal protein gene. Finally, the present invention also contemplates embodiments wherein said deletion is within the adenovirus DNA polymerase and preterminal protein genes.

The present invention further provides cell lines capable of supporting the propagation of Ad virus containing deletions within the E2b region. In one embodiment the invention

25   provides a mammalian cell line stably and constitutively expressing the adenovirus E1 gene products and the adenovirus DNA polymerase. In one embodiment, these cell lines comprise a recombinant adenovirus comprising a deletion within the E2b region, this E2b-deleted recombinant adenovirus being capable of self-propagation in the cell line. The present invention is not limited by the nature of the deletion within the E2b region. In one

30   embodiment, the deletion is within the adenoviral DNA polymerase gene.

The present invention provides cells lines stably expressing E1 proteins and the adenoviral DNA polymerase, wherein the genome of the cell line contains a nucleotide sequence encoding adenovirus DNA polymerase operably linked to a heterologous promoter.

- 5 -

In a particularly preferred embodiment. the cell line is selected from the group consisting of the B-6. B-9. C-1. C-4. C-7. C-13. and C-14 cell lines.

The present invention further provides cell lines which further constitutively express the adenovirus preterminal protein (pTP) gene product (in addition to E1 proteins and DNA polymerase). In one embodiment. these pTP-expressing cell lines comprise a recombinant adenovirus comprising a deletion within the E2b region. the recombinant adenovirus being capable of self-propagation in the pTP-expressing cell line. In a preferred embodiment. the deletion within the E2b region comprises a deletion within the adenoviral preterminal protein gene. In another preferred embodiment. the deletion within the E2b region comprises a deletion within the adenoviral (Ad) DNA polymerase and preterminal protein genes.

In a preferred embodiment. the cell lines coexpressing pTP and Ad DNA polymerase. contain within their genome. a nucleotide sequence encoding adenovirus preterminal protein operably linked to a heterologous promoter. In the invention is not limited by the nature of the heterologous promoter chosen. The art knows well how to select a suitable heterologous promoter to achieve expression in the desired host cell (e.g.. 293 cells or derivative thereof). In a particularly preferred embodiment. the pTP- and Ad polymerase-expressing cell line is selected from the group consisting of the C-1. C-4. C-7. C-13. and C-14 cell lines.

The present invention provides a method of producing infectious recombinant adenovirus particles containing an adenoviral genome containing a deletion within the E2b region. comprising: a) providing: i) a mammalian cell line stably and constitutively expressing the adenovirus E1 gene products and the adenovirus DNA polymerase: ii) a recombinant adenovirus comprising a deletion within the E2b region. the recombinant adenovirus being capable of self-propagation in said cell line: b) introducing the recombinant adenovirus into the cell line under conditions such that the recombinant adenovirus is propagated to form infectious recombinant adenovirus particles: and c) recovering the infectious recombinant adenovirus particles. In a preferred embodiment. the method further comprises d) purifying the recovered infectious recombinant adenovirus particles. In yet another preferred embodiment. the method further comprises e) administering the purified recombinant adenovirus particles to a host which is preferably a mammal and most preferably a human.

In another preferred embodiment the mammalian cell line employed in the above method further constitutively expresses the adenovirus preterminal protein.

The present invention further provides a recombinant plasmid capable of replicating in a bacterial host comprising adenoviral E2b sequences, the E2b sequences containing a deletion within the polymerase gene, the deletion resulting in reduced polymerase activity. The present invention is not limited by the specific deletion employed to reduce polymerase activity. In a preferred embodiment, the deletion comprises a deletion of nucleotides 8772 to 9385 in SEQ ID NO:4. In one preferred embodiment, the recombinant plasmid has the designation pΔpol. In another preferred embodiment, the recombinant plasmid has the designation pBHG11Δpol.

The present invention also provides a recombinant plasmid capable of replicating in a bacterial host comprising adenoviral E2b sequences, the E2b sequences containing a deletion within the preterminal protein gene, the deletion resulting in the inability to express functional preterminal protein without disruption of the VA RNA genes. The present invention is not limited by the specific deletion employed to render the pTP inactive; any deletion within the pTP coding region which does not disrupt the ability to express the Ad VA RNA genes may be employed. In a preferred embodiment, the deletion comprises a deletion of nucleotides 10,705 to 11,134 in SEQ ID NO:4. In one preferred embodiment, the recombinant plasmid has the designation pΔpTP. In another preferred embodiment, the recombinant plasmid has the designation pBHG11ΔpTP.

In a preferred embodiment, the recombinant plasmid containing a deletion with the pTP region further comprises a deletion within the polymerase gene, this deletion resulting in reduced (preferably absent) polymerase activity. The present invention is not limited by the specific deletion employed to inactivate the polymerase and pTP genes. In a preferred embodiment, the deletion comprises a deletion of nucleotides 8,773 to 9586 and 11,067 to 12,513 in SEQ ID NO:4. In one preferred embodiment, the recombinant plasmid has the designation pAXBΔpolΔpTPVARNA+t13. In another preferred embodiment, the recombinant plasmid has the designation pBHG11ΔpolΔpTPVARNA+t13.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a schematic representation of the Ad polymerase expression plasmid pRSV-pol indicating that the Ad2 DNA polymerase sequences are under the transcriptional control of the RSV-LTR/promoter element and are flanked on the 3' end by the SV-40 small t intron and SV-40 polyadenylation addition site.

Figure 1B is a schematic representation of the expression plasmid pRSV-pTP indicating that the Ad2 preterminal protein sequences are under the transcriptional control of the RSV-LTR/promoter element and are flanked on the 3' end by the SV-40 small-t intron and SV-40 polyadenylation signals.

Figure 2 is an ethidium bromide-stained gel depicting the presence of Ad pol DNA sequences in genomic DNA from LP-293 cells and several hygromycin-resistant cell lines. The ~750 bp PCR products are indicated by the arrow.

Figure 3 is an autoradiograph depicting the results of a viral replication-complementation assay analyzing the functional activity of the Ad polymerase protein expressed by LP-293 cells and several hygromycin resistant cell lines. The 8.010 bp HindIII fragments analyzed by densitometry are indicated by an arrow.

Figure 4 is an autoradiograph indicating that cell lines B-6 and C-7 contained a smaller and a larger species of Ad polymerase mRNA while LP-293 derived RNA had no detectable hybridization signal. The location of the two species of Ad polymerase mRNA are indicated relative to the 28S and 18S ribosomal RNAs, and the aberrant transcript expressed by the B-9 cell line is indicated by an arrow.

Figure 5 is an autoradiograph showing which of the cell lines that received the preterminal protein expression plasmid indicated the presence of pRSV-pTP sequences (arrow labelled "pTP") and E1 sequences (arrow labelled "E1").

Figure 6 is an autoradiograph indicating in which of the cell lines transcription of preterminal protein is occurring (arrow labelled "~ 3kb").

Figure 7 is an autoradiograph indicating that the expression of the Ad-polymerase could overcome the replication defect of H5sub100 at non-permissive temperatures.

Figure 8 graphically depicts plaque titre for LP-293, B-6 and C-7 cell lines infected with wtAd5. H5ts36, or H5sub100. and the results demonstrate that the C-7 cell line can be used as a packaging cell line to allow the high level growth of E1. preterminal. and polymerase deleted Ad vectors.

Figure 9 is an autoradiograph showing that the recombinant pol⁻ virus is viable on pol-expressing 293 cells but not on 293 cells which demonstrates that recombinant Ad viruses containing the 612 bp deletion found within pΔpol lack the ability to express Ad polymerase.

Figure 10 is a schematic representation of the structure of pAd5βdys wherein the two inverted adenovirus origins of replication are represented by a left and right inverted terminal repeat (LITR/RITR). P1 and P2 represent location of probes used for Southern blot analysis.

Figure 11 graphically illustrates the total number of transducing adenovirus particles produced (output) per serial passage on 293 cells, total input virus of either the helper (hpAP) or Ad5βdys, and the total number of cells used in each infection.

Figures 12A-B show Southern blot analyses of viral DNA from lysates 3, 6, 9 and 12, digested with the restriction enzymes BssHII, NruI and EcoRV. For the analyses, fragments from the C terminus of *mus musculus* dystrophin cDNA (A) or the N terminus of *E. coli* β-galactosidase (B) were labeled with dCTP^{32} and used as probes.

Figures 13A-B show the physical separation of Ad5βdys from hpAP virions at the third (A) and final (B) stages of CsCl purification.

Figure 14 graphically depicts the level of contamination of Ad5βdys EAMs by hpAP virions obtained from the final stage of CsCl purification as measured by β-galactosidase and alkaline phosphatase expression. The ratio of the two types of virions - Ad5βdys EAMs (LacZ) or hpAP (AP) in each fraction is indicated in the lower graph.

Figures 15A-B are western blot (immunoblot) analyses of protein extracts from *mdx* myoblasts and myotubes demonstrating the expression of β-galactosidase (A) and dystrophin (B) in cells infected with Ad5βdys EAMs.

Figures 16A-C depict immunofluorescence of dystrophin expression in wild type MM14 myotubes (A), uninfected *mdx* (B) and infected *mdx* myotubes (C).

Figure 17 is a schematic representation of the MCK/lacZ constructs tested to determine what portion of the ~ 3.3 kb DNA fragment containing the enhancer/promoter of the MCK gene is capable of directing high levels of expression of linked genes in muscle cells.

Figure 18 is a schematic representation of a GFP/β-gal reporter construct suitable for assaying the expression of *Cre* recombinase in mammalian cells.

Figure 19 is a schematic representation of the recombination event between the *loxP* shuttle vector and the Ad5dl7001 genome.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor thereof. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained. The term "gene" encompasses both cDNA and genomic forms of a given gene.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers

5   to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

The term "oligonucleotide" as used herein is defined as a molecule comprised of two

10   or more deoxyribonucleotides or ribonucleotides, usually more than three (3), and typically more than ten (10) and up to one hundred (100) or more (although preferably between twenty and thirty). The exact size will depend on many factors, which in turn depends on the ultimate function or use of the oligonucleotide. The oligonucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, or a

15   combination thereof.

As used herein, the term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals,

20   termination signals, *etc.* (defined *infra*).

Transcriptional control signals in eucaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription [Maniatis, T. *et al.*, Science 236:1237 (1987)]. Promoter and enhancer elements have been isolated from a variety of

25   eukaryotic sources including genes in yeast, insect and mammalian cells and viruses (analogous control elements, *i.e.*, promoters, are also found in procaryotes). The selection of a particular promoter and enhancer depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types [for review see Voss, S.D. *et al.*, Trends

30   Biochem. Sci., 11:287 (1986) and Maniatis, T. *et al.*, *supra* (1987)].

The term "recombinant DNA vector" as used herein refers to DNA sequences containing a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism (e.g.

mammal). DNA sequences necessary for expression in procaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, polyadenlyation signals and enhancers.

The terms "in operable combination", "in operable order" and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "genetic cassette" as used herein refers to a fragment or segment of DNA containing a particular grouping of genetic elements. The cassette can be removed and inserted into a vector or plasmid as a single unit. A plasmid backbone refers to a piece of DNA containing at least plasmid origin of replication and a selectable marker gene (e.g., an antibiotic resistance gene) which allows for selection of bacterial hosts containing the plasmid; the plasmid backbone may also include a polylinker region to facilitate the insertion of genetic elements within the plasmid. When a particular plasmid is modified to contain non-plasmid elements (e.g., insertion of Ad sequences and/or a eukaryotic gene of interest linked to a promoter element), the plasmid sequences are referred to as the plasmid backbone.

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3' end of one oligonucleotide points towards the 5' end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide.

The term "primer" refers to an oligonucleotide which is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically.

A primer is selected to be "substantially" complementary to a strand of specific sequence of the template. A primer must be sufficiently complementary to hybridize with a template strand for primer elongation to occur. A primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be

5   attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the template to hybridize and thereby form a template primer complex for synthesis of the extension product of the primer.

10   "Hybridization" methods involve the annealing of a complementary sequence to the target nucleic acid (the sequence to be detected). The ability of two polymers of nucleic acid containing complementary sequences to find each other and anneal through base pairing interaction is a well-recognized phenomenon. The initial observations of the "hybridization" process by Marmur and Lane, *Proc. Natl. Acad. Sci. USA* 46:453 (1960) and Doty *et al.*,

15   *Proc. Natl. Acad. Sci. USA* 46:461 (1960) have been followed by the refinement of this process into an essential tool of modern biology.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not

20   commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide,

25   base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

Stability of a nucleic acid duplex is measured by the melting temperature, or "T_m." The T_m of a particular nucleic acid duplex under specified conditions is the temperature at which on average half of the base pairs have disassociated. The equation for calculating the

30   T_m of nucleic acids is well known in the art.

The term "probe" as used herein refers to a labeled oligonucleotide which forms a duplex structure with a sequence in another nucleic acid, due to complementarity of at least one sequence in the probe with a sequence in the other nucleic acid.

The term "label" as used herein refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like.

The terms "nucleic acid substrate" and nucleic acid template" are used herein interchangeably and refer to a nucleic acid molecule which may comprise single- or double-stranded DNA or RNA.

"Oligonucleotide primers matching or complementary to a gene sequence" refers to oligonucleotide primers capable of facilitating the template-dependent synthesis of single or double-stranded nucleic acids. Oligonucleotide primers matching or complementary to a gene sequence may be used in PCRs, RT-PCRs and the like.

A "consensus gene sequence" refers to a gene sequence which is derived by comparison of two or more gene sequences and which describes the nucleotides most often present in a given segment of the genes; the consensus sequence is the canonical sequence.

The term "polymorphic locus" is a locus present in a population which shows variation between members of the population (*i.e.*, the most common allele has a frequency of less than 0.95). In contrast, a "monomorphic locus" is a genetic locus at little or no variations seen between members of the population (generally taken to be a locus at which the most common allele exceeds a frequency of 0.95 in the gene pool of the population).

The term "microorganism" as used herein means an organism too small to be observed with the unaided eye and includes, but is not limited to bacteria, viruses, protozoans, fungi, and ciliates.

The term "microbial gene sequences" refers to gene sequences derived from a microorganism.

The term "bacteria" refers to any bacterial species including eubacterial and archaebacterial species.

The term "virus" refers to obligate, ultramicroscopic, intracellular parasites incapable of autonomous replication (*i.e.*, replication requires the use of the host cell's machinery). Adenoviruses, as noted above, are double-stranded DNA viruses. The left and right inverted terminal repeats (ITRs) are short elements located at the 5` and 3` termini of the linear Ad genome, respectively and are required for replication of the viral DNA. The left ITR is located between 1-130 bp in the Ad genome (also referred to as 0-0.5 mu). The right ITR is

located from ~3.7500 bp to the end of the genome (also referred to as 99.5-100 mu). The two ITRs are inverted repeats of each other. For clarity. the left ITR or 5' end is sued define the 5' and 3' ends of the ITRs. The 5' end of the left ITR is located at the extreme 5' end of the linear adenoviral genome: picturing the left ITR (LITR) as an arrow extending from the 5'

5   end of the genome. the tail of the 5' ITR is located at mu 0 and the head of the left ITR is located at ~ 0.5 mu (further the tail of the left ITR is referred to as the 5' end of the left ITR and the head of the left ITR is referred to as the 3' end of the left ITR). The tail of the right or 3' ITR is located at mu 100 and the head of the rigt ITR is located at ~ mu 99.5: the head of the right ITR is referred to as the 5' end of the right ITR and the tail of the right ITR is

10   referred to as the 3' end of the right ITR (RITR). In the linear Ad genome. the ITRs face each other with the head of each ITR pointing inward toward the bulk of the genome. When arranged in a "tail to tail orientation" the tails of each ITR (which comprise the 5' end of the LITR and the 3' end of the RITR) are located in proximity to one another while the heads of each ITR are separated and face outward (see for example. the arrangement of the ITRs in the

15   EAM shown in Figure 10 herein). The "adenovirus packaging sequence" refers to the Ψ sequence which comprises five (AI-AV) packaging signals and is required for encapsidation of the mature linear genome: the packaging signals are located from ~194 to 358 bp in the Ad genome (about 0.5-1.0 mu).

The phrase "at least one adenovirus gene coding region" refers to a nucleotide

20   sequence containing more than one adenovirus gene coding gene. A "helper adenovirus" or "helper virus" refers to an adenovirus which is replication-competent in a particular host cell (the host may provide Ad gene products such as E1 proteins). this replication-competent virus is used to supply *in trans* functions (*e.g.*. proteins) which are lacking in a second replication-incompetent virus: the first replication-competent virus is said to "help" the second

25   replication-incompetent virus thereby permitting the propagation of the second viral genome in the cell containing the helper and second viruses.

The term "containing a deletion within the E2b region" refers to a deletion of at least one basepair (preferably more than one bp and preferably at least 100 and most preferably more than 300 bp) within the E2b region of the adenovirus genome. An E2b deletion is a

30   deletion that prevents expression of at least one E2b gene product and encompasses deletions within exons encoding portions of E2b-specific proteins as well as deletions within promoter and leader sequences.

An "adenovirus minichromosome" refers to a linear molecule of DNA containing the Ad ITRs on each end which is generated from a plasmid containing the ITRs and one or more gene of interest. The term "encapsidated adenovirus minichromosome" or "EAM" refers to an adenovirus minichromosome which has been packaged or encapsidated into a viral particle; plasmids containing the Ad ITRs and the packaging signal are shown herein to produce EAMs. When used herein. "recovering" encapsidated adenovirus minichromosomes refers to the collection of EAMs from a cell containing an EAM plasmid and a helper virus; this cell will direct the encapsidation of the minichromosome to produce EAMs. The EAMs may be recovered from these cells by lysis of the cell (e.g., freeze-thawing) and pelleting of the cell debris to a cell extract as described in Example 1 (Ex. 1 describes the recovery of Ad virus from a cell, but the same technique is used to recover EAMs from a cell). "Purifying" such minichromosomes refers to the isolation of the recovered EAMs in a more concentrated form (relative to the cell lysate) on a density gradient as described in Example 7; purification of recovered EAMs permits the physical separation of the EAM from any helper virus (if present).

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation. DEAE-dextran-mediated transfection. polybrene-mediated transfection. electroporation. microinjection. liposome fusion. lipofection. protoplast fusion. retroviral infection. and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

As used herein. the term "gene of interest" refers to a gene inserted into a vector or plasmid whose expression is desired in a host cell. Genes of interest include genes having therapeutic value as well as reporter genes. A variety of such genes are contemplated. including genes of interest encoding a protein which provides a therapeutic function (such as the dystrophin gene. which is capable of correcting the defect seen in the muscle of MD patients). the utrophin gene. the CFTR gene (capable of correcting the defect seen in cystitic fibrosis patients). etc..

The term "reporter gene" indicates a gene sequence that encodes a reporter molecule (including an enzyme). A "reporter molecule" is detectable in any detection system. including. but not limited to enzyme (e.g., ELISA. as well as enzyme-based histochemical

assays), fluorescent, radioactive, and luminescent systems. In one embodiment, the present invention contemplates the *E. coli* β-galactosidase gene (available from Pharmacia Biotech, Pistacataway, NJ), green fluorescent protein (GFP) (commercially available from Clontech, Palo Alto, CA), the human placental alkaline phosphatase gene, the chloramphenicol acetyltransferase (CAT) gene; other reporter genes are known to the art and may be employed.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

DESCRIPTION OF THE INVENTION

The present invention provides improved adenovirus vectors for the delivery of recombinant genes to cells *in vitro* and *in vivo*. As noted above, the present invention contemplates two approaches to improving adenovirus vectors. The first approach generally contemplates a recombinant plasmid containing the minimal region of the Ad genome required for replication and packaging (*i.e.*, the left and right ITR and the packaging or Ψ sequence) along with one or more genes of interest; this recombinant plasmid is packaged into an encapsidated adenovirus minichromosome (EAM) when grown in parallel with an E1-deleted helper virus in a cell line expressing the E1 proteins (*e.g.*, 293 cells). The recombinant adenoviral minichromosome is preferentially packaged. To prevent the packaging of the helper virus, a helper virus containing *loxP* sequences flanking the Ψ sequence is employed in conjunction with 293 cells expressing *Cre* recombinase: *Cre-loxP* mediated recombination removes the packaging sequence form the helper genome thereby preventing packaging of the helper during the production of EAMs. In the second approach, "damaged" or "deleted" adenoviruses containing deletions within the E2b region are employed. While the "damaged" adenovirus is capable of self-propagation in a packaging cell line expressing the appropriate E2b protein(s), the E2b-deleted recombinant adenovirus are incapable of replicating and expressing late viral gene products outside of the packaging cell line.

In one embodiment, the self-propagating recombinant adenoviruses containing deletions in the E2b region of the adenovirus genome. In another embodiment, "gutted"

viruses are contempla⬤these viruses lack all viral coding re⬤ns. In addition. packaging cell lines co-expressing E1 and E2b gene products are provided which allow the production of infectious recombinant virus containing deletions in the E1 and E2b regions without the use of helper virus.

The Description of the Invention is divided into the following sections: I. Self-Propagating Adenovirus Vectors: II. Packaging Cell Lines: and III. Encapsidated Adenoviral Minichromosomes.

I.   Self-Propagating Adenovirus Vectors

Self-propagating adenovirus (Ad) vectors have been extensively utilized to deliver foreign genes to a great variety of cell types *in vitro* and *in vivo*. "Self-propagating viruses" are those which can be produced by transfection of a single piece of DNA (the recombinant viral genome) into a single packaging cell line to produce infectious virus: self-propagating viruses do not require the use of helper virus for propagation.

Existing Ad vectors have been shown to be problematic *in vivo*. This is due in part because current or first generation Ad vectors are deleted for only the early region 1 (E1) genes. These vectors are crippled in their ability to replicate normally without the trans-complementation of E1 functions provided by human 293 cells. a packaging cell line [ATCC CRL 1573: Graham *et al.* (1977) J. Gen. Virol. 36:59]. Unfortunately. with the use of high titres of E1 deleted vectors. and the fact that there are E1-like factors present in many cell types. E1 deleted vectors can overcome the block to replication and express other viral gene products [Imperiale et al. (1984) Mol. Cell Biol. 4:867: Nevins (1981) Cell 26:213: and Gaynor and Berk (1983) Cell 33:683]. The expression of viral proteins in the infected target cells elicits a swift host immune response. that is largely T-cell mediated [Yang and Wilson (1995) J. Immunol. 155:2564 and Yang *et al.* (1994) Proc. Natl. Acad. Sci. USA 91:4407]. The transduced cells are subsequently eliminated. along with the transferred foreign gene. In immuno-incompetent animals. Ad delivered genes can be expressed for periods of up to one year [Yang *et al.* (1994). supra: Vincent *et al.* (1993) Nature Genetics 5:130: and Yang *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:7257].

Another shortcoming of first generation Ad vectors is that a single recombination event between the genome of an Ad vector and the integrated E1 sequences present in 293 cells can generate replication competent Ad (RCA). which can readily contaminate viral stocks.

In order to further cripple viral protein expression, and also to decrease the frequency of generating RCA, the present invention provides Ad vectors containing deletions in the E2b region. Propagation of these E2b-deleted Ad vectors requires cell lines which express the deleted E2b gene products. The present invention provides such packaging cell lines and for the first time demonstrates that the E2b gene products, DNA polymerase and preterminal protein, can be constitutively expressed in 293 cells along with the E1 gene products. With every gene that can be constitutively expressed in 293 cells comes the opportunity to generate new versions of Ad vectors deleted for the respective genes. This has immediate benefits; increased carrying capacity, since the combined coding sequences of the polymerase and preterminal proteins that can be theoretically deleted approaches 4.6 kb and a decreased incidence of RCA generation, since two or more independent recombination events would be required to generate RCA. Therefore, the novel E1, Ad polymerase and preterminal protein expressing cell lines of the present invention enable the propagation of Ad vectors with a carrying capacity approaching 13 kb, without the need for a contaminating helper virus [Mitani *et al.* (1995) Proc. Natl. Acad. Sci. USA 92:3854]. In addition, when genes critical to the viral life cycle are deleted (*e.g.*, the E2b genes), a further crippling of Ad to replicate and express other viral gene proteins occurs. This decreases immune recognition of virally infected cells, and allows for extended durations of foreign gene expression. The most important attribute of E1, polymerase, and preterminal protein deleted vectors, however, is their inability to express the respective proteins, as well as a predicted lack of expression of most of the viral structural proteins. For example, the major late promoter (MLP) of Ad is responsible for transcription of the late structural proteins L1 through L5 [Doerfler, In *Adenovirus DNA, The Viral Genome and Its Expression* (Martinus Nijhoff Publishing Boston, 1986)]. Though the MLP is minimally active prior to Ad genome replication, the rest of the late genes get transcribed and translated from the MLP only *after* viral genome replication has occurred [Thomas and Mathews (1980) Cell 22:523]. This cis-dependent activation of late gene transcription is a feature of DNA viruses in general, such as in the growth of polyoma and SV-40. The polymerase and preterminal proteins are *absolutely* required for Ad replication (unlike the E4 or protein IX proteins) and thus their deletion is extremely detrimental to Ad vector late gene expression.

II.   Packaging Cell Lines Constitutively Expressing E2b Gene
       Products

The present invention addresses the limitations of current or first generation Ad
vectors by isolating novel 293 cell lines coexpressing critical viral gene functions. The
present invention describes the isolation and characterization of 293 cell lines capable of
constitutively expressing the Ad polymerase protein. In addition. the present invention
describes the isolation of 293 cells which not only express the E1 and polymerase proteins.
but also the Ad-preterminal protein. The isolation of cell lines coexpressing the E1. Ad
polymerase and preterminal proteins demonstrates that three genes critical to the life cycle of
Ad can be constitutively coexpressed. without toxicity.

In order to delete critical genes from self-propagating Ad vectors. the proteins encoded
by the targeted genes have to first be coexpressed in 293 cells along with the E1 proteins.
Therefore. only those proteins which are non-toxic when coexpressed constitutively (or toxic
proteins inducibly-expressed) can be utilized. Coexpression in 293 cells of the E1 and E4
genes has been demonstrated (utilizing inducible. not constitutive. promoters) [Yeh *et al.*
(1996) J. Virol. 70:559; Wang *et al.* (1995) Gene Therapy 2:775; and Gorziglia *et al.* (1996)
J. Virol. 70:4173]. The E1 and protein IX genes (a virion structural protein) have been
coexpressed [Caravokyri and Leppard (1995) J. Virol. 69:6627]. and coexpression of the E1.
E4. and protein IX genes has also been described [Krougliak and Graham (1995) Hum. Gene
Ther. 6:1575].

The present invention provides for the first time. cell lines coexpressing E1 and E2b
gene products. The E2b region encodes the viral replication proteins which are absolutely
required for Ad genome replication [Doerfler. *supra* and Pronk *et al.* (1992) Chromosoma
102:S39-S45]. The present invention provides 293 cells which constitutively express the 140
kD Ad-polymerase. While other researchers have reported the isolation of 293 cells which
express the Ad-preterminal protein utilizing an inducible promoter [Schaack *et al.* (1995) J.
Virol. 69:4079]. the present invention is the first to demonstrate the high-level. constitutive
coexpression of the E1. polymerase. and preterminal proteins in 293 cells. without toxicity.
These novel cell lines permit the propagation of novel Ad vectors deleted for the E1,
polymerase. and preterminal proteins.

III.　Encapsidated Adenoviral Minichromosomes

The present invention also provides encapsidated adenovirus minichromosome (EAM) consisting of an infectious encapsidated linear genome containing Ad origins of replication. packaging signal elements. a reporter gene (e.g.. a β-galactosidase reporter gene cassette) and a gene of interest (e.g.. a full length (14 kb) dystrophin cDNA regulated by a muscle specific enhancer/promoter). EAMs are generated by cotransfecting 293 cells with supercoiled plasmid DNA (e.g.. pAd5βdys) containing an embedded inverted origin of replication (and the remaining above elements) together with linear DNA from E1-deleted virions expressing human placental alkaline phosphatase (hpAP) (a helper virus). All proteins necessary for the generation of EAMs are provided *in trans* from the hpAP virions and the two can be separated from each other on equilibrium CsCl gradients. These EAMs are useful for gene transfer to a variety of cell types both *in vitro* and *in vivo*.

Adenovirus-mediated gene transfer to muscle is a promising technology for gene therapy of Duchenne muscular dystrophy (DMD). However. currently available recombinant adenovirus vectors have several limitations. including a limited cloning capacity of ~8.5 kb. and the induction of a host immune response that leads to transient gene expression of 3 to 4 weeks in immunocompetent animals. Gene therapy for DMD could benefit from the development of adenoviral vectors with an increased cloning capacity to accommodate a full length (~14 kb) dystrophin cDNA. This increased capacity should also accommodate gene regulatory elements to achieve expression of transduced genes in a tissue-specific manner. Additional vector modifications that eliminate adenoviral genes. expression of which is associated with development of a host immune response. might greatly increase long term expression of virally delivered genes *in vivo*. The constructed encapsidated adenovirus minichromosomes of the present invention are capable of delivering up to 35 kb of non-viral exogenous DNA. These minichromosomes are derived from bacterial plasmids containing two fused inverted adenovirus origins of replication embedded in a circular genome. the adenovirus packaging signals. a βgalactosidase reporter gene and a full length dystrophin cDNA regulated by a muscle specific enhancer/promoter. The encapsidated minichromosomes are propagated *in vitro* by *trans*-complementation with a replication defective (E1+E3 deleted) helper virus. These minichromosomes can be propagated to high titer (>10^8/ml) and purified on CsCl gradients due to their buoyancy difference relative to helper virus. These vectors are able to transduce myogenic cell cultures and express dystrophin in myotubes. These results

demonstrate that encapsidated adenovirus minichromosomes are useful for gene transfer to muscle and other tissues.

The present invention further provides methods for modifying the above-described EAM system to enable the generation of high titer stocks of EAMs with minimal helper virus contamination. Preferably the EAM stocks contain helper virus representing less than 1%, preferably less than 0.1% and most preferably less than 0.01% (including 0.0%) of the final viral isolate.

The amount of helper virus present in the EAM preparations is reduced in two ways. The first is by selectively controlling the relative packaging efficiency of the helper virus versus the EAM virus. The *Cre-loxP* excision method is employed to remove the packaging signals from the helper virus thereby preventing the packaging of the helper virus used to provide in trans viral proteins for the encapsidation of the recombinant adenovirus minichromosomes. The second approach to reducing or eliminating helper virus in EAM stocks is the use of improve physical methods for separating EAM from helper virus.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: M (molar); mM (millimolar); μM (micromolar); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); mu or m.u. (map unit); g (gravity); gm (grams); mg (milligrams); μg (micrograms); pg (picograms); L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); hr (hour); min (minute); msec (millisecond); °C (degrees Centigrade); AMP (adenosine 5'-monophosphate); cDNA (copy or complimentary DNA); DTT (dithiotheritol); ddH$_2$O (double distilled water); dNTP (deoxyribonucleotide triphosphate); rNTP (ribonucleotide triphosphate); ddNTP (dideoxyribonucleotide triphosphate); bp (base pair); kb (kilo base pair); TLC (thin layer chromatography); tRNA (transfer RNA); nt (nucleotide); VRC (vanadyl ribonucleoside complex); RNase (ribonuclease); DNase (deoxyribonuclease); poly A (polyriboadenylic acid); PBS (phosphate buffered saline); OD (optical density); HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid]); HBS (HEPES buffered saline); SDS (sodium dodecyl sulfate); Tris-HCl (tris[Hydroxymethyl]aminomethane-hydrochloride); rpm (revolutions per minute); ligation buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 10 mM

dithiothreitol. 25 μg/ml bovine serum albumin. and 26 μM NAD+. and pH 7.8): EGTA
(ethylene glycol-bis(β-aminoethyl ether) N. N. N'. N'-tetraacetic acid): EDTA
(ethylenediaminetetracetic acid): ELISA (enzyme linked immunosorbant assay): LB (Luria-
Bertani broth: 10 g tryptone. 5 g yeast extract. and 10 g NaCl per liter. pH adjusted to 7.5

5   with 1N NaOH): superbroth (12 g tryptone. 24 g yeast extract. 5 g glycerol. 3.8 g KH$_2$PO$_4$
and 12.5 g. K$_2$HPO$_4$ per liter): DMEM (Dulbecco's modified Eagle's medium): ABI (Applied
Biosystems Inc.. Foster City. CA): Amersham (Amersham Corporation. Arlington Heights.
IL): ATCC (American Type Culture Collection. Rockville. MY): Beckman (Beckman
Instruments Inc.. Fullerton CA): BM (Boehringer Mannheim Biochemicals. Indianapolis. IN):

10   Bio-101 (Bio-101. Vista. CA): BioRad (BioRad. Richmond. CA): Brinkmann (Brinkmann
Instruments Inc. Wesbury. NY): BRL. Gibco BRL and Life Technologies (Bethesda Research
Laboratories. Life Technologies Inc.. Gaithersburg. MD): CRI (Collaborative Research Inc.
Bedford. MA): Eastman Kodak (Eastman Kodak Co.. Rochester. NY): Eppendorf (Eppendorf.
Eppendorf North America. Inc.. Madison. WI): Falcon (Becton Dickenson Labware. Lincoln

15   Park. NJ): IBI (International Biotechnologies. Inc.. New Haven. CT): ICN (ICN Biomedicals.
Inc.. Costa Mesa. CA): Invitrogen (Invitrogen. San Diego. CA): New Brunswick (New
Brunswick Scientific Co. Inc.. Edison. NJ): NEB (New England BioLabs Inc.. Beverly. MA):
NEN (Du Pont NEN Products. Boston. MA): Pharmacia (Pharmacia LKB Gaithersburg. MD):
Promega (Promega Corporation. Madison. WI): Stratagene (Stratagene Cloning Systems. La

20   Jolla. CA): UVP (UVP. Inc.. San Gabreil. CA); USB (United States Biochemical Corp..
Cleveland. OH): and Whatman (Whatman Lab. Products Inc. Clifton. NJ).

Unless otherwise indicated. all restriction enzymes and DNA modifying enzymes were
obtained from New England Biolabs (NEB) and used according to the manufacturers
directions.

25

EXAMPLE 1

Generation Of Packaging Cell Lines That Coexpress
The Adenovirus E1 And DNA Polymerase Proteins

30   In this example. packaging cell lines coexpressing Ad E1 and polymerase proteins
were described. These cell lines were shown to support the replication and growth of H5ts36.
an Ad with a temperature-sensitive mutation of the Ad polymerase protein. These

polymerase-expressing packaging cell lines can be used to prepare Ad vectors deleted for the E1 and polymerase functions.

a)   Tissue Culture And Virus Growth

LP-293 cells (Microbix Biosystems. Toronto) were grown and serially passaged as suggested by the supplier.

Plaque assays were performed in 60 mm dishes containing cell monolayers at ~ 90% confluency. The appropriate virus dilution in a 2% DMEM solution was dripped onto the cells. and the plates incubated at the appropriate temperature for one hour. The virus containing media was aspirated. the monolayer was overlaid with 10 mls of a pre-warmed EMEM agar overlay solution (0.8% Noble agar. 4% fetal calf serum. and antibiotics) and allowed to solidify. After the appropriate incubation time (usually 7 days for incubations at 38.5°C and 10-12 days for incubations at 32°C). five mls of the agar-containing solution containing 1.3% neutral red was overlaid onto the infected dishes and plaques were counted the next day. An aliquot of the virus H5ts36 [Freimuth and Ginsberg (1986) Proc. Natl. Acad. Sci. USA 83:7816] was utilized to produce high titre stocks after infection of 293 cells at 32°C. H5ts36 is an Ad5-derived virus defective for viral replication at the nonpermissive temperature [Miller and Williams (1987) J. Virol. 61:3630].

The infected cells were harvested after the onset of extensive cytopathic effect. pelleted by centrifugation and resuspended in 10 mM Tris-Cl. pH 8.0. The lysate was freeze-thawed three times and centrifuged to remove the cell debris. The cleared lysate was applied to CsCl$_2$ step gradients (heavy CsCl at density of 1.45 g/ml. the light CsCl at density of 1.20 g/ml). ultracentrifuged. and purified using standard techniques [Graham and Prevec (1991) In *Methods in Molecular Biology, Vol 7: Gene Transfer and Expression Protocols.* Murray (ed.). Humana Press. Clifton. NJ. pp. 109-128]. The concentration of plaque forming units (pfu) of this stock was determined at 32°C as described above. Virion DNA was extracted from the high titre stock by pronase digestion. phenol-chloroform extraction. and ethanol precipitation. The leakiness of this stock was found to be <1 in 2000 pfu at the non-permissive temperature. consistent with previous reports [Miller and Williams (1987). *supra*].

b)   Plasmids

The expression plasmid pRSV-pol [Zhao and Padmanabhan (1988) Cell 55:1005] contains sequences encoding the Ad2 polymerase mRNA (including the start codon from the

- 23 -

exon at map unit 39) under the transcriptional control of the Rous Sarcoma Virus LTR/promoter element; the Ad2 DNA polymerase sequences are flanked on the 3' end by the SV-40 small t intron and SV-40 polyadenylation addition site (see Fig. 1A). Figure 1A provides a schematic representation of the Ad polymerase expression plasmid pRSV-pol.

5   pRSV-pol includes the initiator methionine and amino-terminal peptides encoded by the exon at m.u. 39 of the Ad genome. The location of the PCR primers p602a and p2158c. the two *Sca*I 1 kb probes utilized for Northern analyses. the polymerase terminator codon. and the polyadenylation site of the IVa2 gene (at 11.2 m.u. of the Ad genome) are indicated.

The expression plasmid pRSV-pTP [Zhao and Padmanabhan (1988). *supra*] contains

10   sequences encoding the Ad2 preterminal protein (including the amino terminal peptides encoded by the exon at map unit 39 of the Ad genome) under the transcriptional control of the Rous Sarcoma Virus LTR/promoter element; the pTP sequences are flanked on the 3' end by the SV-40 small-t intron and SV-40 polyadenylation signals (see Figure 1B for a schematic of pRSV-PTP). Figure 1B also shows the location of the *Eco*RV subfragment utilized as a

15   probe in the genomic DNA and cellular RNA evaluations. as well as the initiator methionine codon present from map unit 39 in the Ad5 genome. The locations of the H5*in*190 and H5*sub*100 insertions are shown relative to the preterminal protein open-reading frame. The following abbreviations are used in Figure 1: ORF. open reading frame: small-t. small tumor antigen. and m.u.. map unit.

20   pCEP4 is a plasmid containing a hygromycin expression cassette (Invitrogen).

pFG140 (Microbix Biosystems Inc. Toronto. Ontario) is a plasmid containing sequences derived from Ad5*dl*309 which contain a deletion/substitution in the E3 region [Jones and Shek (1979) Cell 17:683]. pFG140 is infectious in single transfections of 293 cells and is used as a control for transfection efficiency.

25

c)   Transfection Of 293 Cells

LP-293 cells were cotransfected with *Bam*HI linearized pRSV-pol and *Bam*HI linearized pCEP4 at a molar ratio of 10:1 using a standard $CaPO_4$ precipitation method [Sambrook *et al.* (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor

30   Press. Plainview. NY. pp.16.33-16.36]. In addition. 293 cells were cotransfected with or with *Bam*HI linearized pRSV-pol. *Bam*HI linearized pRSV-pTP and pCEP using a molar ratio of 10:1 (non-selectable:selectable plasmids).

Forty-eight hours after transfection. the cells were passaged into media containing hygromycin at 100 μg/mL. Individual hygromycin resistant colonies were isolated and expanded.

d) Isolation Of Ad Polymerase Expressing 293 Cell Lines

Twenty hygromycin resistant cell lines were expanded and screened for the ability to express the Ad polymerase protein. Initially. the individual cell lines were assayed for the ability to support growth of the viral polymerase mutant H5ts36 using the plaque assay described in section a) above. It was speculated that constitutive expression of the wild type Ad polymerase protein in a clonal population of 293 cells should allow the growth of H5ts36 at 38.5°C. However. it was unclear if constitutive expression of the Ad polymerase would be toxic when coexpressed with the E1 proteins present in 293 cells. Similar toxicity problems have been observed with the Ad ssDBP. and the pTP [Klessig *et al.* (1984) Mol. Cell. Biol. 4:1354 and Schaack *et al.* (1995) J. Virol. 69:4079].

Of the twenty hygromycin resistant cell lines isolated. seven were able to support plaque formation with H5ts36 at the non-permissive temperature. unlike the parental LP-293 cells: these cell lines were named B-6. B-9. C-1. C-4. C-7. C-13 and C-14 (see Table 1) (the B-6 and B-9 cell lines received only the pRSV-pol and CEP4 plasmids; C-1. C-4. C-7. C-13 and C-14 cell lines received the pRSV-pol. pRSV-pTP and CEP4 plasmids). For the results shown in Table 1. dishes (60 mm) of near confluent cells of each cell line were infected with the same dilution of H5ts36 at the temperature indicated. overlaid with agar media. and stained for plaques as outlined in section a. Passage number refers to the number of serial passages after initial transfection with the plasmid pRSV-pol.

The cell line B-6 produced plaques one day earlier than the other Ad polymerase-expressing cell lines. which may reflect increased polymerase expression (see below). Cell line B-9 demonstrated an increased doubling time whereas each of the other cell lines displayed no growth disadvantages relative to the parental LP-293 cells. As shown in Table 1. even after multiple passages (in some instances up to four months of serial passaging) the cells were still capable of H5ts36 plaque formation at the non-permissive temperature. indicating that the RSV-LTR/promoter remained active for extended periods of time. However. the cell lines B-9 and C-13 displayed a decreased ability to plaque the virus at 32°C as well as at 38.5°C. suggesting that a global viral complementation defect had occurred in

these cell lines after extended passaging. The remaining cell lines screened at later passages
demonstrated no such defect, even after 20 passages (e.g., cell line B-6, Table 1).

TABLE 1

Plaquing Ability Of H5ts36 At The Non-Permissive Temperature Utilizing 293-Ad Polymerase Expressing
Cell Lines

Cell Line	Passage Number	Number Of Plaques At:	
		32.0°C	38.5°C
293	-	>500	0
B-6	9	>500	>500
	20	>500	>500
B-9	5	>500	>500
	14	90	18
C-1	5	>500	>500
	13	>500	>500
C-4	5	>500	>500
	14	>500	>500
C-7	5	>500	>500
	14	>500	>500
C-13	5	>500	>500
	27	120	4
C-14	5	>500	>500
	27	>500	370

c)   **Genomic Analysis Of Ad Polymerase-Expressing Cell Lines**

Genomic DNA from LP-293 cells and each of the seven cell lines able to complement
H5ts36 at 38.5°C were analyzed by PCR for the presence of pRSV-pol derived sequences.
Genomic DNA from LP-293 cells and the hygromycin resistant cell lines were harvested
using standard protocols (Sambrook et al., supra) and 200 ng of DNA from each cell line was
analyzed by PCR in a solution containing 2 ng/mL of primers p602a and p2158c, 10 mM
TrisHCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001% gelatin. The forward primer,
p602a [5'-TTCATTTTATGTTTCAGGTTC AGGG-3' (SEQ ID NO:2)] is located in the SV-
40 polyadenylation sequence. The reverse primer p2158c [5'-
TTACCGCCACACTCGCAGGG-3' (SEQ ID NO:3)] is Ad-sequence specific with the 5'
nucleotide located at position 3394 of the Ad 5 genome [numbering according to Doerfler

(1986) *Adenovirus DNA. The Viral Genome and Its Expression*. Nijhoff. Boston. MA. pp. 1-95].

PCR was performed with a Perkin Elmer 9600 Thermocycler utilizing the following cycling parameters: initial denaturation at 94°C for 3 min. 3 cycles of denaturation at 94°C for 30 sec. annealing at 50°C for 30 sec. and extension at 72°C for 60 sec. followed by another 27 cycles with an increased annealing temperature at 56°C. with a final extension at 72°C for 10 minutes. PCR products were separated on a 1.0 % agarose gel and visualized with ethidium bromide staining (Figure 2). A 1 kb ladder (Gibco-BRL) was used as a size marker. and the plasmid pRSV-pol was used as a positive control. The ~750 bp PCR products are indicated by an arrow in Figure 2.

As shown in Figure 2. all cell lines capable of H5ts36 plaque formation at 38.5°C contained the Ad pol DNA sequences. whereas the LP-293 cells did not yield any amplification product with these primers. This result demonstrates that each of the selected cell lines stably co-integrated not only the hygromycin resistance plasmid pCEP4. but also pRSV-pol.

f) Complementation Of The Replication Defect Of H5ts36 By Ad Polymerase Expressing Cell Lines

The C to T transition at position 7623 of the H5ts36 genome alters the DNA binding affinity of the Ad polymerase protein. rendering it defective for viral replication at non-permissive temperatures [Chen *et al.* (1994) Virology 205:364: Miller and Williams (1987) J. Virol. 61:3630: and Wilkie *et al.* (1973) Virology 51:499]. To analyze the functional activity of the Ad polymerase protein expressed by each of the packaging cell lines. a viral replication-complementation assay was performed. LP-293 cells or the hygromycin resistant cell lines were seeded onto 60 mM dishes at densities of 2.5-3.0 x 10⁶ per dish. infected with H5ts36 at a multiplicity of infection (MOI) of 10. and incubated for 24 hours at 38.5°C. or 48 hours at 32°C. Total DNA was harvested from each plate. then 2 μg of each sample were digested with *Hind*III. separated on a 1.0% agarose gel. transferred to a nylon membrane. and hybridized with ³²P-labeled H5ts36 virion DNA. Densitometric analysis of the 8.010 bp *Hind*III fragment in each lane was performed on a phophoroimager (Molecular Dynamics) utilizing a gel image processing system (IP Lab Version 1.5. Sunnyvale. CA).

The resulting autoradiograph is shown in Figure 3. In Figure 3. the lane marked "Std." (standard) contains 1 µg of HindIII-digested H5ts36 virion DNA. The 8.010 bp HindIII fragments analyzed by densitometry are indicated by an arrow.

As shown in Figure 3. H5ts36 had a diminished ability to replicate in LP-293 cells at the non-permissive temperature. In contrast. all seven of the previously selected cell lines were able to support replication of H5ts36 virion DNA at 38.5°C to levels approaching those occurring in LP-293 cells at 32°C.

A densitometric analysis of the amount of H5ts36 viral DNA replicated in each of the cell lines at permissive and nonpermissive temperatures is presented in Table 2 below. For this assay. the relative amounts of the 8.010 bp HindIII fragment were compared. The relative levels of H5ts36 virion DNA replication determined by densitometric analysis of the 8.010 bp HindIII fragment isolated from each of the cell line DNA samples. The surface area of the 8.010 bp fragment in 293 cells incubated at 38.5°C was designated as 1. and includes some replicated H5ts36 virion DNA. The numbers in each column represent the ratio between the densities of the 8.010 bp fragment isolated in the indicated cell line and the density of the same band present in LP-293 cells at 38.5°C.

As shown in Table 2. the levels of replication at the permissive temperature were all within four-fold of each other. regardless of which cell line was analyzed. but at the non-permissive temperature LP-293 cells reveal the H5ts36 replication defect. The viral bands that were present in the LP-293 DNA sample at 38.C represented input virion DNA as well as low level replication of H5ts36 DNA. which is generated due to the leakiness of the ts mutation at the high MOI utilized in this experiment. The Ad polymerase-expressing cell lines were all found to be capable of augmenting

TABLE 2

Densitometric Analysis Of H5ts36 Replication

Cell Line	Ratios Of 8.010 bp HindIII Fragment Generated At:	
	32.0°C	38.5°C
LP-293	42.6	1.0
B-6	27.3	57.2
B-9	69.0	15.8
C-1	113.6	34.1
C-4	100.2	65.5
C-7	114.9	75.0
C-13	43.1	42.2
C-14	46.7	27.8

H5ts36 genome replication. Although one cell line (B-9) allowed H5ts36 replication to levels only 16 fold greater than LP-293 cells, this was the same cell line that was observed to display poor growth properties. Each of the remaining Ad polymerase-expressing cell lines allowed substantially greater replication of H5ts36 at non-permissive temperatures, compared to LP-293 cells (Table 2). An enhancement of replication up to 75 fold above that of LP-293 cells was observed with the cell line C-7 at 38.5°C. A substantially more rapid onset of viral cytopathic effect in Ad polymerase-expressing cell lines was observed at either temperature. These estimates of H5ts36 replication-complementation are conservative, since they have not been adjusted for the low level replication of H5ts36 at 38.5°C [Miller and Williams (1987), *supra*]. The leakiness of the H5ts36 mutation could potentially be overcome with the use of a virus deleted for the Ad polymerase gene.

g) RNA Analysis Of Ad Polymerase-Expressing Cell Lines

Total RNA was extracted from each of the cell lines using the RNAzol method (Teltest, Inc., Friendswood, TX 77546; Chomczynski and Sacchi (1987) Anal. Biochem. 162:156]. Fifteen micrograms of RNA from each cell line was electrophoresed on a 0.8% agarose-formaldehyde gel, and transferred to a Nytran membrane (Schleicher & Schuell) by blotting. The filter was UV crosslinked, and analyzed by probing with the two ^{32}P-labeled 1 kb ScaI subfragments of Ad which span positions 6095-8105 of the Ad5 genome (see Figure 1A). These two ScaI subfragments of the Ad genome are complimentary to the 5' end of the Ad polymerase mRNA. The resulting autoradiograph is shown in Figure 4. In Figure 4, the

location of the smaller and larger species of Ad polymerase mRNA are indicated relative to the 28S and 18S ribosomal RNAs. The aberrant transcript expressed by the B-9 cell line is indicated by an arrow.

The results shown in Figure 4 revealed that the RNA derived from cell lines B-6 and C-7 contained two species of RNA, estimated to be ~4800 and ~7000 nt in length, while LP-293 derived RNA had no detectable hybridization signal. The presence of two polymerase RNA species suggests that the polyadenylation signal of the Ad IVa2 gene (present in the Ad-pol construct, see Fig. 1A) is being utilized by the cell RNA processing machinery, in addition to the SV-40 polyadenylation signal. Similar analysis of RNA derived from the cell lines C-1, C-4, C-13, and C-14 also detected the same two transcripts as those detected in the RNA of cell lines B-6 and C-7, but at decreased levels, suggesting that even low levels of Ad polymerase mRNA expression can allow for the efficient replication of polymerase mutants such as H5ts36. The cell line B-6 expressed high levels of polymerase transcript and can plaque H5ts36 one day earlier than the other cell lines at 38.5°C, suggesting a causal relationship. It is interesting to note that the two polymerase transcripts are also detected in RNA isolated from cell line B-9, but substantial amounts of a larger RNA transcript (size > 10 kb) is also present (see Figure 4). The high level production of the aberrant message may be related to the increased doubling time previously noted in this cell line.

h) Transfectability Of Ad Polymerase-Expressing Cell Lines

The ability of Ad polymerase-expressing 293 cell lines to support production of H5ts36 virions after transfection with H5ts36 genome DNA was examined as follows. 293 cells as well as hygromycin resistant cell lines were grown to near confluency on 60 mm dishes and transfected with either 3 μg of purified H5ts36 virion DNA, or with 3.5 μg of the plasmid pFG140 (Microbix Biosystems), using the cationic lipid Lipofectamine (Gibco-BRL). Cells that received the H5ts36 virion DNA were incubated at 32°C for 14 days, or 38.5°C for 10 days. The pFG140 transfected cells were incubated at 37.5°C for 10 days. All plates were then stained with the neutral red agar overlay and plaques were counted the next day. The results are shown in Table 3.

TABLE 3

Transfection Efficiency Of Ad pol-Expressing Cell Lines

Cell line	Number Of Plaques At:	
	32.0°C	38.5°C
LP-293	>500	0
B-6	>500	~500
B-9	n.d.*	n.d.
C-1	n.d.	>500
C-4	n.d.	>500
C-7	n.d.	>500
C-13	n.d.	100
C-14	n.d.	>500

n.d. = not determined.

The results shown in Table 3 demonstrated that transfection of H5ts36 DNA at the non-permissive temperature allows for ample plaque production in all of the Ad polymerase-expressing cell lines tested. unlike the parental LP-293 cells. Cell line C-13 was at passage number 29. and demonstrated a somewhat decreased ability to generate plaques at this extended passage number. These same cell lines are also capable of producing plaques when transfected with the plasmid pFG140. a plasmid capable of producing infectious. E1 dependent Ad upon transfection of the parental 293 cells [Ghosh-Choudhury (1986) Gene 50:161]. These observations suggest that the Ad polymerase expressing cell lines should be useful for the production of second generation Ad vectors deleted not only for the E1 genes. but also for the polymerase gene. As shown below in Examples 2 and 3. this is indeed the case.

EXAMPLE 2

Isolation And Characterization Of Packaging Cell Lines That
Coexpress The Adenovirus E1. DNA Polymerase And Preterminal Proteins

In Example 1. packaging cell lines coexpressing Ad E1 and polymerase proteins were described. These cell lines were shown to support the replication and growth of H5ts36. an Ad with a temperature-sensitive mutation of the Ad polymerase protein. These polymerase-

expressing packaging cell lines can be used to prepare Ad vectors deleted for the E1 and polymerase functions. In this example, 293 cells cotransfected with both Ad polymerase and preterminal protein expression plasmids are characterized. Cell lines co-expressing the Ad E1, polymerase and preterminal proteins can be used to prepare Ad vectors deleted for the E1, polymerase and preterminal protein (pTP) functions.

a)   Tissue Culture And Virus Propagation

The use of LP-293 cells (Microbix Biosystems Inc., Toronto), Ad-polymerase expressing cell lines, and plaquing efficiency assays of Ad viruses was conducted as described in Example 1. All cells were maintained in 10% fetal bovine serum supplemented DMEM media (GIBCO) in the presence of antibiotics. The virus H5*sub*100 [Freimuth and Ginsberg (1986) Proc. Natl. Acad. Sci. USA 83:7816] has a temperature sensitive (ts) mutation caused by a three base pair insertion within the amino terminus of the preterminal protein, in addition to a deletion of the E1 sequences (see Figure 1B). H5*sub*100 was propagated and titred at 32.0°C in LP-293 cells; the leakiness of this stock was less than 1 per 1000 plaque-forming units (pfu) at the nonpermissive temperature of 38.5°C. A lower titer cell lysate contaning the virus H5*in*190 (which contains a 12 base-pair insertion within the carboxy-terminus of the preterminal protein as well as a deletion of the E1 region, see Figure 1B) was provided by Dr. P. Freimuth [Freimuth and Ginsberg (1986), *supra*]. The polymerase and preterminal protein expressing cell lines were always maintained in media supplemented with hygromycin (Sigma) at 100 μg/mL.

b)   Isolation Of Ad Polymerase And Preterminal Protein
Expressing 293 Cells

The C-1, C-4, C-7, C-13, and C-14 cell lines (Ex. 1), which had been cotransfected with pRSV-pol, pRSV-pTP and CEP4, were screened for presence of pTP sequences and for the ability to support the growth of H5ts36 (ts for the Ad-polymerase), H5*in*190, and H5*sub*100 using plaque assays as described in Example 1.

i)   Analysis Of Genomic DNA And Cellular RNA

Cell lines that had received the preterminal protein expression plasmid were screen for the presence of pRSV-pTP sequences and E1 sequences. Total DNA was isolated from the LP-293. B-6 (transfected with pRSV-pol only) or C-7 (cotransfected with pRSV-pol and pRSV-pTP) cell lines. two micrograms (µg) of each DNA was codigested with the restriction enzymes *Xba*I and *Bam*HI. electrophoretically separated in a 0.6% agarose gel. and transferred onto a nylon membrane. The membrane was UV crosslinked. probed with both a 1.8 kb *Bln*I-*Xba*I fragment (spans the E1 coding region) isolated from the plasmid pFG140. and a 1.8kb *Eco*RV subfragment of Ad serotype 5 (spans the preterminal protein coding sequences; see Fig. 1B). both of which were random-primer radiolabeled with ^{32}P to a specific activity greater than 3.0×10^8 cpm/µg. The membrane was subsequently exposed to X-ray film with enhancement by a fluorescent screen. The resulting autoradiograph is shown in Figure 5.

In Figure 5. the preterminal specific sequences migrated as an ~11.0 kb DNA fragment while the E1 containing band migrated as a 2.3 kb DNA fragment. No hybridization of either probe to DNA isolated from either the LP-293 or B-6 cell lines was observed As shown in Figure 5. only the C-7 cell line genomic DNA had preterminal coding sequences. unlike the parental LP-293 cells. or the Ad-polymerase expressing B-6 cells. In addition. all cell lines had E1 specific sequences present at nearly equivalent amounts. demonstrating that the selection design has not caused the loss of the E1 sequences originally present in the LP-293 cells. The results presented in Example 1 demonstrated that both the B-6 and C-7 cell lines contain polymerase specific sequences within their genomes. unlike the parental LP-293 cells.

To confirm that transcription of preterminal protein was occurring. total RNA was isolated from each of the cell lines. transferred to nylon membranes. and probed to detect preterminal protein-specific mRNA transcripts as follows. Total cellular RNA was isolated from the respective cell lines and 15 µg of total RNA from each cell line was transferred to nylon membranes. The membranes were probed with the 1.8 kb *Eco*RV radiolabeled subfragment of Ad5 (see Fig. 1B) complementary to the preterminal protein coding region. The resulting autoradiograph is shown in Figure 6.

As shown in Figure 6. a single mRNA of the expected size (~3 kb in length) is detected only in RNA derived from the C-7 cell line. No hybridization was detected in lanes containing RNA derived from the LP-293 or B-6 cell lines. In Example 1. it was demonstrated that the C-7 cell line also expresses high levels of the Ad polymerase mRNA.

Thus. the C-7 cell line constitutively expresses both the Ad polymerase and preterminal protein mRNAs along with E1 transcripts.

ii)   Plaquing Efficiency Of pTP Mutants On pTP-Expressing Cell Lines

The C-7 cell line was screened for the ability to transcomplement the growth of preterminal mutant viruses. The virus H5in190 (contains a 12 base pair insertion located within the carboxy-terminus of the preterminal protein) has been shown to have a severe growth and replication defect. producing less than 10 plaque-forming units per cell [Freimuth and Ginsberg (1986) Proc. Natl. Acad. Sci. USA 83:7816]. The results are summarized in Table 4 below. For the results shown in Table 4, LP-293. B-6. or C-7 cells were seeded at a density of $2.0-2.5 \times 10^6$ cells per plate. The cells were infected with limiting dilutions of lysates derived from the preterminal protein-mutant viruses H5in190 or H5sub100. incubated at 38.5°C. and plaques counted after six days. As shown in Table 4. only the C-7 cell line could allow efficient plaque formation of H5in190 at 38.5°C (the H5in190 lysate used to infect the cells was of a low titer. relative to the high titer H5sub100 stock). while both the B-6 and C-7 cell lines had nearly equivalent plaquing efficiencies when H5sub100 was utilized as the infecting virus.

TABLE 4

Plaquing Efficiency Of Preterminal Protein-Mutant Viruses

Virus	Mutation Location	Plaque Titres (pfu/ml)		
		LP-293	B-6	C-7
H5in190	carboxy-terminus	$<1 \times 10^2$	$<1 \times 10^2$	1.4×10^5
H5sub100	amino-terminus	$<1 \times 10^4$	9.0×10^5	4.5×10^8

As shown in Table 4. when equivalent dilutions of H5in190 were utilized. the plaquing efficiency of the C-7 cell line was at least 100-fold greater than that of the B-6 or LP-293 cells. This result demonstrated that the C-7 cell line produces a functional preterminal protein. capable of trans-complementing the defect of the H5in190 derived preterminal protein.

The cell lines were next screened for the ability to trans-complement with the temperature-sensitive virus. H5sub100. at nonpermissive temperatures. H5sub100 has a codon insertion mutation within the amino-terminus of the preterminal protein. as well as an E1 deletion. The mutation is responsible both for a temperature sensitive growth defect. as well

as a replication defect [reimuth and Ginsberg (1986). *supra* and Schaack *et al.* (1995) J. Virol. 69:4079]. The plaquing efficiency of the cell line C-7 was found to be at least 1000 fold greater than that of the LP-293 cells (at non-permissive temperatures) (see Table 4). Interestingly, the cell line B-6 was also capable of producing large numbers of H5sub100 derived plaques at 38.5°C, even though it does not express any preterminal protein. This result suggested that the high level expression of the polymerase protein was allowing plaque formation of H5sub100 in the B-6 cell line. To examine this possibility, the nature of H5sub100 growth in the various cell lines was examined.

c) Complementation Of The Replication And Growth Defects Of H5sub100

The cell lines B-6 and C-7 were shown to overcome the replication defect of H5ts36 (Ex. 1). Since the preterminal and polymerase proteins are known to physically interact with each other [Zhao and Padmanabhan (1988) Cell 55:1005], we investigated whether the expression of the Ad-polymerase could overcome the replication defect of H5sub100 at non-permissive temperatures using the following replication-complementation assay.

LP-293, B-6, or C-7 cells were seeded onto 60 mM dishes at a density of 2×10^6 cells per dish and infected the next day with H5sub100 at a multiplicity of infection (MOI) of 0.25, and incubated at 38.5°C for 16 hours or 32.0°C for 40 hours. The cells from each infected plate were then harvested and total DNA extracted as described in Example 1. Four micrograms of each DNA sample was digested with *Hind*III, electrophoresed through a 0.7% agarose gel, transferred to a nylon membrane, and probed with ^{32}P-labeled H5ts36 virion DNA. The resulting autoradiograph is shown in Figure 7. As seen in Figure 7, the H5sub100 replication defect when grown in LP-293 cells at 38.5°C is seen; this defect is not present when the virus is grown at the same temperature in either B-6 or C-7 cells.

The results depicted in Figure 7 demonstrates that both cell lines B-6 and C-7 could trans-complement the replication defect of H5sub100. This result demonstrated that the expression of the Ad polymerase in B-6 cells was able to overcome the preterminal protein-mediated replication defect of H5sub100. While not limiting the present invention to any particular mechanism, the ability of Ad polymerase to overcome the preterminal protein-mediated replication defect of H5sub100 may be due to a direct physical interaction of the polymerase with the amino-terminus of the H5sub100-derived preterminal protein.

In support of this hypothesis, it has also been demonstrated that the H5sub100 replication defect can be overcome when LP-293 cells were infected with a 100-fold greater amount of H5sub100. However, complementation of the H5sub100 replication defect is not sufficient to overcome the growth defect of H5sub100, since temperature shift-up experiments

5   have demonstrated that the H5sub100 growth defect is not dependent upon viral replication [Schaack *et al.* (1995), *supra*]. Therefore, the overexpression of the Ad-polymerase must have allowed a very low level but detectable production of infectious H5sub100 particles in the B-6 cell line. The reduced growth of H5sub100 is therefore not due to a replication defect, but rather some other critical activity that the preterminal protein has a role in, such as

10   augmention of viral transcription by association with the nuclear matrix [Schaack and Shenk (1989) Curr. Top. Microbiol. Immunol. 144:185 and Hauser and Chamberlain (1996) J. Endo. 149:373]. This was confirmed by assessing the ability of the C-7 cell line to overcome the growth defect of H5sub100 utilizing one-step growth assays performed as follows.

        Each of the cell lines (LP-293, B-6 and C-7) were seeded onto 60 mm dishes at 2.0 x

15   10⁶ cells/dish. The cell lines were infected at an MOI of 4 with each of the appropriate viruses (wtAd5, H5ts36, or H5sub100), and incubated at 38.5°C for 40 hours. The total amount of infectious virions produced in each 60 mm dish was released from the cell lysates by three cycles of freeze-thawing, and the titer was then determined by limiting dilution and plaque assay on B-6 cells at 38.5°C. The results are summarized in Figure 8.

20   The results shown in Figure 8, demonstrated that even though the B-6 cell line allowed normal replication and plaque formation of H5sub100 at 38.5°C (in fact, B-6 cells were utilized to determine the plaque titres depicted in Figure 8) they could not allow high level growth of H5sub100 and only produced titres of H5sub100 equivalent to that produced by the LP-293 cells. The C-7 cell line produced 100 fold more virus than the LP-293 or B-6 cells,

25   see Figure 8. Encouragingly, the titre of H5sub100 produced by the C-7 cells approached titres produced by LP-293 cells infected with wild-type virus, Ad5. When the H5sub100 virions produced from infection of the C-7 cells were used to infect LP-293 cells at 38.5°C, all virus produced retained the *ts* mutation (*i.e.*, at least a 1000 fold drop in pfu was detected when LP-293 cells were respectively infected at 38.5°C vs. 32.0°C). This finding effectively

30   rules out the theoretical possibility that the H5sub100 input virus genomes recombined with the preterminal protein sequences present in the C-7 cells.

        In addition, the C-7 cell line allowed the high level growth of H5ts36, demonstrating that adequate amounts of the Ad-polymerase protein were also being expressed. The C-7 cell

line was capable of trans-complementing the growth of both H5ts36 and H5sub100 after 4 months of serial passaging, demonstrating that the coexpression of the E1, preterminal, and polymerase proteins was not toxic.

These results demonstrate that the constitutive expression of both the polymerase and preterminal proteins is not detrimental to normal virus production, which might have occurred if one or both of the proteins had to be expressed only during a narrow time period during the Ad life cycle. In summary, these results demonstrated that the C-7 cell line can be used as a packaging cell line to allow the high level growth of E1, preterminal, and polymerase deleted Ad vectors.

EXAMPLE 3

Production Of Adenovirus Vectors

Deleted For E1 And Polymerase Functions

In order to produce an Ad vectors deleted for E1 and polymerase functions, a small, frame-shifting deletion was introduced into the Ad-pol gene contained within an E1-deleted Ad genome. The plasmid pBHG11 (Microbix) was used as the source of an E1-deleted Ad genome. pBHG11 contains a deletion of Ad5 sequences from bp 188 to bp 1339 (0.5-3.7 m.u.): this deletion removes the packaging signals as well as E1 sequences. pBHG11 also contains a large deletion within the E3 region (bp 27865 to bp 30995: 77.5-86.2 m.u.). The nucleotide sequence of pBHG11 is listed in SEQ ID NO:4 [for cross-corrleation between the pBHG11 sequence and the Ad5 genome (SEQ ID NO:1), it is noted that nucleotide 8,773 in pBHG11 is equivalent to nucleotide 7,269 in Ad5].

pBHG11 was chosen to provide the Ad backbone because this plasmid contains a large deletion within the E3 region (77.5 to 86.2 m.u.) and therefore vectors derived from this plasmid permit the insertion of large pieces of foreign DNA. A large cloning capacity is important when the pol⁻ vectors is to be used to transfer a large gene such as the dystrophin gene (cDNA = 13.6 kb). However, the majority of genes are not this large and therefore other Ad backbones containing smaller deletions within the E3 region (e.g., pBHG10 which contains a deletion between 78.3 to 85.8 m.u.; Microbix) may be employed for the construction of pol⁻ vectors using the strategy outlined below.

a)   Construction Of A Plasmid Containing A Portion Of The
Adenovirus Genome Containing The Polymerase Gene

A fragment of the Ad genome containing the pol gene located on pBHG11 was
subcloned to create pBSA-XB. Due to the large size of the Ad genome. this intermediate
5   plasmid was constructed to facilitate the introduction of a deletion within the pol gene the pol
deletion. pBSA-XB was constructed as follows. The polylinker region of pBluescript
(Stratagene) was modified to include additional restriction enzyme recognition sites (the
sequence of the modified polylinker is provided in SEQ ID NO:5: the remainder of
pBluescript was not altered); the resulting plasmid was termed pBSX. pBHG11 was digested
10   with *Xba*l and *Bam*Hl and the 20.223 kb fragment containing the pol and pTP coding regions
(E2b region) was inserted into pBSX digested with *Xba*l and *Bam*HI to generate pBSA-XB.

b)   Construction Of pBHG11Δpol

A deletion was introduced into the pol coding region contained within pBSA-XB in
15   such a manner that other key viral elements were not disturbed (*e.g.*, the major late promoter.
the tripartite leader sequences. the pTP gene and other leader sequences critical for normal
virus viability). The deletion of the pol sequences was carried out as follows. pBSA-XB was
digested with *Bsp*EI and the ends were filled in using T4 DNA polymerase. The *Bsp*EI-
digested. T4 polymerase filled DNA was then digested with *Bam*HI and the 8809 bp
20   *Bam*HI/*Bsp*EI(filled) fragment was isolated as follows. The treated DNA was run on a 0.6%
agarose gel (TAE buffer) and the 8809 bp fragment was excised from the gel and purified
using a QIAEX Gel Extraction Kit according to the manufacturer's instructions (OIAGEN.
Chatsworth. CA).

A second aliquot of pBSA-XB DNA was digested with *Bsp*HI and the ends were filled
25   in with T4 DNA polymerase. The *Bsp*HI-digested. T4 polymerase filled DNA was then
digested with *Bam*HI and the 13.679 bp *Bam*HI/*Bsp*HI(filled) fragment was isolated as
described above.

The purified 8809 bp *Bam*HI/*Bsp*EI(filled) fragment and the purified 13.679 bp
*Bam*HI/*Bsp*HI(filled) fragment were ligated to generate pΔpol. pΔpol contains a 612 bp
30   deletion within the pol gene (bp 8772 to 9385: numbering relative to that of pBHG11) and
lacks the 11.4 kb *Bam*HI fragment containing the right arm of the Ad genome found within
pBHG11.

To provide the right arm of the Ad genome, pΔpol was digested with BamHI followed by treatment with calf intestinal alkaline phosphatase. pBHG11 was digested with BamHI and the 11.4 kb fragment was isolated and purified using a QIAEX Gel Extraction Kit as described above. The purified 11.4 kb BamHI fragment was ligated to the BamHI/phosphatased pΔpol to generate pBHG11Δpol. Proper construction of pBHG11Δpol was confirmed restriction digestion (HindIII).

c)   Rescue And Propagation Of Ad5Δpol Virus

The Ad genome contained within pBHG11Δpol lacks the packaging signals. Therefore, in order to recover virus containing the 612 bp deletion within the pol gene from pBHG11Δpol, this plasmid must be cotransfected into packaging cells along with DNA that provides a source of the Ad packaging signals. The Ad packaging signals may be provided by wild-type or mutant Ad viral DNA or alternatively may be provided using a shuttle vector which contains the left-end Ad5 sequences including the packaging signals such as pΔE1sp1A, pΔE1sp1B (Microbix) or pAdBglII (pAdBglII is a standard shuttle vector which contains 0-1 m.u. and 9-16 m.u. of the adenovirus genome).

To rescue virus, pBHG11Δpol was co-transfected with Ad5dl7001 viral DNA . Ad5dl7001 contains a deletion in the E3 region: the E3 deletion contained within Ad5dl7001 is smaller than the deletion contained within the Ad genome contained within pBHG11. It is not necessary that Ad5dl7001 be used to recover virus; other adenoviruses, including wild-type adenoviruses, may be used to rescue of virus from pBHG11Δpol.

It has been reported that the generation of recombinant Ads is more efficient if Ad DNA-terminal protein complex (TPC) is employed in conjunction with a plasmid containing the desired deletion [Miyake et al. (1996) Proc. Natl. Acad. Sci. USA 93:1320]. Accordingly, Ad5dl7001-TPC were prepared as described [Miyake et al. (1996). supra]. Briefly, purified Ad5dl7001 virions (purified through an isopycnic CsCl gradient centered at 1.34 g/ml) were lysed by the addition of an equal volume of 8 M guanidine hydrochloride. The released Ad5dl7001 DNA-TPC was then purified through a buoyant density gradient of 2.8 M CsCl/4 M guanidine hydrochloride by centrifugation for 16 hr at 55,000 rpm in a VTi65 rotor (Beckman). Gradient fractions containing Ad5dl7001 DNA-TPC were identified using an ethidium bromide spot test and then pooled, dialyzed extensively against TE buffer. BSA was then added to a final concentration of 0.5 mg/ml and aliquots were stored at -80°C. The

Ad5dl7001 DNA-TPC was then digested with ScaI and then gel-filtered through a Sephadex G-50 spin column.

One hundred nanograms of the digested Ad5dl7001 DNA-TPC was mixed with 5 μg of pBHG11Δpol and used to transfect pol-expressing 293 cells (i.e.. C-7). Approximately 10 days post-transfection. plaques were picked. The recombinant viruses were plaque purified and propagated using standard techniques (Graham and Prevac. supra). Viral DNA was isolated. digested with restriction enzymes and subjected to Southern blotting analysis to determine the organization of the recovered viruses. Two forms of virus containing the 612 bp pol deletion were recovered and termed Ad5ΔpolΔE3I and Ad5ΔpolΔE3II. One form of recombinant pol⁻ virus recovered. Ad5ΔpolΔE3I. underwent a double recombination event with the Ad5dl7001 sequences and contains the E3 deletion contained within Ad5dl7001 at the right end of the genome. The second form of recombinant pol⁻ virus. Ad5ΔpolΔE3II. retained pBHG11 sequences at the right end of the genome (i.e.. contained the E3 deletion found within pBHG11). These results demonstrate the production of a recombinant Ad vector containing a deletion within the pol gene.

d)   Characterization Of The E1+, pol- Viruses

To demonstrate that the deletion contained within these two pol⁻ viruses renders the virus incapable of producing functional polymerase. Ad5ΔpolΔE3I was used to infect 293 cells and pol-expressing 293 cells (B-6 and C-7 cell lines) and a viral replication-complementation assay was performed as described in Example 1e. Briefly. 293. B-6 and C-7 cells were seeded onto 60 mm dishes at a density of 2 x 106 cells/dish and infected with H5sub100 or Ad5ΔpolΔE3I at an MOI of 0.25. The infected cells were then incubated at 37°C or 38.5°C for 16 hours. or at 32.0°C for 40 hours. Cells from each infected plate were then harvested and total DNA was extracted. Four micrograms of each DNA sample was digested with HindIII. electrophoresised through an agarose gel. transferred to a nylon membrane and probed with ³²P-labeled adenoviral DNA. The resulting autograph is shown in Figure 9. In Figure 9. each panel shows. from left to right. DNA extracted from 293. B-6 and C-7 cells. respectively infected with either H5sub100 or Ad5ΔpolΔE3I (labeled Ad5ΔPOL in Fig. 9).

As shown in Figure 9. the recombinant pol⁻ virus was found to be viable on pol-expressing 293 cells but not on 293 cells. These results demonstrates that recombinant Ad viruses containing the 612 bp deletion found within pΔpol lack the ability to express Ad

polymerase. These results also demonstrate that B-6 and C-7 cells efficiently complement the Δpol found within Ad5ΔpolΔE3I and Ad5ΔpolΔE3II. In addition, these results show that replication of the ts pTP mutant H5sub100 can be complemented by high level expression of the Ad polymerase with or without co-expression of pTP.

Because the expression of early genes is required for the expression of the late gene products, the ability of recombinant viruses containing the Δpol deletion to direct the expression of late gene products was examined. 293 and C-7 cells were infected with Ad5ΔpolΔE3I and 24 hours after infection cell extracts were prepared. The cell extracts were serially diluted and examined for the expression of the fiber protein (a late gene product) by immunoblot analysis. The immunoblot was performed as described in Example 3C with the exception that the primary antibody used was an anti-fiber antibody (FIBER-KNOB obtained from Robert Gerard, University of Texas Southwestern Medical School). The results of this immunoblotting analysis revealed that no fiber protein was detected from 293 cells infected with Ad5ΔpolΔE3I (at any dilutuion of the cell extract). In contrast, even a 1:1000 dilution of cell extract prepared from C-7 cells infected with Ad5ΔpolΔE3I produced a visible band on the immunoblot. Therefore, the pol deletion contained within Ad5ΔpolΔE3I resulted in a greater than 1000-fold decrease in fiber production.

The above results demonstrate that polymerase gene sequences can be deleted from the virus and that the resulting deleted virus will only grow on cells producing Ad-polymerase *in trans*. Using the pol-expressing cell lines described herein (*e.g.*, B-6 and C-7), large quantities of the pol⁻ viruses can be prepared. A dramatic shut-down in growth and late gene expression is seen when cells which do not express Ad polymerase are infected with the pol⁻ viruses.

e)   **Generation Of E1⁻, Pol⁻ Ad Vectors**

Ad5*dl*7001 used above to recuse virus containing the polymerase deletion is an E1-containing virus. The presence of E1 sequences on the recombinant pol⁻ viruses is undesirable when the recombinant virus is to be used to transfer genes into the tissues of animals: the E1 region encodes the transforming genes and such viruses replicate extremely well *in vivo* leading to an immune response directed against cells infected with the E1-containing virus.

E1⁻ viruses containing the above-described polymerase deletion are generated as follows. pBHG11Δpol is cotransfected into pol-expressing 293 cells (*e.g.*, B-6 or C-7) along

with a shuttle vector containing the left-end Ad5 sequences including the packaging signals.
Suitable shuttle vectors include pΔE1sp1A (Microbix), pΔE1sp1B (Microbix) or pAdBglII.
The gene of interest is inserted into the polylinker region of the shuttle vector and this
plasmid is then cotransfected into B-6 or C-7 cells along with pBHG11Δpol to generate a

5　　recombinant E1⁻, pol⁻ Ad vector containing the gene of interest.

EXAMPLE 4

Production Of Adenovirus Vectors
Deleted For E1 And Preterminal Protein Functions

10

In order to produce an Ad vectors deleted for E1 and preterminal protein functions, a
small deletion was introduced into the Ad preterminal protein (pTP) gene contained within an
E1-deleted Ad genome. The plasmid pBHG11 (Microbix) was used as the source of an E1-
deleted Ad genome to maximize the cloning capacity of the resulting pTP⁻ vector. However,

15　　other Ad backbones containing smaller deletions within the E3 region (e.g., pBHG10 which
contains a deletion between 78.3 to 85.8 m.u.; Microbix) may be employed for the
construction of pTP⁻ vectors using the strategy outlined below.

a)　　Construction Of pΔpTP

20　　A deletion was introduced into the pTP coding region contained within pBSA-XB (Ex.
3) in such a manner that other key viral elements were not disturbed (e.g., the tripartite leader
sequences, the i-leader sequences, the VA-RNA I and II genes, the 55 kD gene and the pol
gene). The deletion of the pTP sequences was carried out as follows. pBSA-XB was
digested with XbaI and EcoRV and the 7.875 kb fragment was isolated as described (Ex. 3).

25　　Another aliquot of pBSA-XB was digested with MunI and the ends were filled in using T4
DNA polymerase. The MunI-digested, T4 polymerase filled DNA was then digested with
XbaI and the 14.894 kb XbaI/MunI(filled) fragment was isolated as described (Ex. 3). The
7.875 kb MunI fragment and the 14.894 kb XbaI/MunI(filled) fragment were ligated together
to generate pΔpTP.

30

b)   Construction Of pBHG11ΔpTP

pΔpol contains a 429 bp deletion within the pTP gene (bp 10.705 to 11.134: numbering relative to that of pBHG11) and lacks the 11.4 kb BamHI fragment containing the right arm of the Ad genome found within pBHG11.

To provide the right arm of the Ad genome. pΔpTP was digested with BamHI followed by treatment with shrimp alkaline phosphatase (SAP: U.S. Biochemicals. Cleveland, OH). pBHG11 was digested with BamHI and the 11.4 kb fragment was isolated and purified using a QIAEX Gel Extraction Kit as described (Ex. 3). The purified 11.4 kb BamHI fragment was ligated to the BamHI/phosphatased pΔpTP to generate pBHG11ΔpTP. Proper construction of pBHG11ΔpTP was confirmed restriction digestion.

c)   Rescue And Propagation Of Ad Vectors Containing The pTP Deletion

The Ad genome contained within pBHG11ΔpTP lacks the Ad packaging signals. Therefore. in order to recover virus containing the 612 bp deletion within the pol gene from pBHG11Δpol. this plasmid must be cotransfected into packaging cells along with DNA that provides a source of the Ad packaging signals. The Ad packaging signals may be provided by wild-type or mutant Ad viral DNA or alternatively may be provided using a shuttle vector which contains the left-end Ad5 sequences including the packaging signals such as pΔE1sp1A. pΔE1sp1B (Microbix) or pAdBglII.

Recombinant Ad vectors containing the pTP deletion which contain a deletion within the E3 region are generated by cotransfection of pBHG11ΔpTP (the gene of interest is inserted into the unique PacI site of pBHG11ΔpTP) with a E3-deleted Ad virus such as Ad5dl700 into pTP-expressing 293 cells (e.g.. C-7); viral DNA-TPC are utilized as described above in Example 3.

Recombinant vectors containing the pTP deletion which also contain deletions within the E1 and E3 regions are generated by cotransfection of pBHG11ΔpTP into pTP-expressing 293 cells (e.g.. C-7) along with a shuttle vector containing the left-end Ad5 sequences including the packaging signals. Suitable shuttle vectors include pΔE1sp1A (Microbix). pΔE1sp1B (Microbix) or pAdBglII. The gene of interest is inserted into the polylinker region of the shuttle vector and this plasmid is then cotransfected into B-6 or C-7 cells along with pBHG11ΔpTP to generate a recombinant E1⁻. pTP⁻ Ad vector containing the gene of interest.

EXAMPLE 5

Production Of Adenovirus Vectors Deleted For

E1. Polymerase And Preterminal Protein Functions

5   In order to produce an Ad vectors deleted for E1. polymerase and preterminal protein

functions. a deletion encompassing pol and pTP gene sequences was introduced into the Ad

sequences contained within an E1-deleted Ad genome. The plasmid pBHG11ΔE4 was used

as the source of an E1-deleted Ad genome to maximize the cloning capacity of the resulting

pol⁻. pTP⁻ vector. pBHG11ΔE4 is a modified form of BHG11 which contains a deletion of

10   all E4 genes except for the E4 ORF 6; the E4 region was deleted to create more room for the

insertion of a gene of interest and to further disable the virus. However. other E1⁻ Ad

backbones. such as pBHG11 and pBHG1 (Microbix; pBHG10 contains a smaller deletion

within the E3 region as compared to pBHG11). may be employed for the construction of

pol⁻. pTP⁻ vectors using the strategy outlined below.

15   Due to the complexity of the cloning steps required to introduce a 2.3 kb deletion that

removes portions of both the pTP and pol genes. this deletion was generated using several

steps as detailed below.

a)   Construction Of pAXBΔpolΔpTPVARNA+t13

20   In order to create a plasmid containing Ad sequences that have a deletion within the

pol and pTP genes. pAXBΔpolΔpTPVARNA+t13 was constructed as follows.

pBSA-XB was digested with *Bsp*EI and the 18 kb fragment was isolated and

recircularized to create pAXBΔpolΔpTP; this plasmid contains a deletion of the sequences

contained between the *Bsp*EI sites located at 8.773 and 12.513 (numbering relative to

25   pBHG11).

A fragment encoding the VA-RNA3 sequence and the third leader of the tri-partite

leader sequence was prepared using the PCR as follows. The PCR was carried out in a

solution containing H5ts36 virion DNA (any Ad DNA. including wild-type Ad. may be used).

2 ng/mL of primers 4005E and 4006E. 10 mM Tris HCl. pH 8.3. 50 mM KCl. 1.5 mM

30   MgCl₂. 0.001% gelatin and *Pfu* polymerase. The forward primer. 4005E. [5'-

TGCCGCAGCACCGGATGCATC-3' (SEQ ID NO:6)] contains sequences complementary to

residues 12.551 to 12.571 of pBHG11 (SEQ ID NO:4). The reverse primer. 4006E. [5'-

GCG<u>TCCGGA</u>GGCTGCCATG CGGCAGGG-3' (SEQ ID NO:7)] is complementary to

residues 11.091 to 11.108 of pBHG11 (SEQ ID NO:4) as well as a *Bsp*EI site (underlined). The predicted sequence of the ~1.6 kb PCR product is listed in SEQ ID NO:8.

PCR was performed with a Perkin Elmer 9600 Thermocycler utilizing the following cycling parameters: initial denaturation at 94°C for 3 min. 3 cycles of denaturation at 94°C for 30 sec. annealing at 50°C for 30 sec. and extension at 72°C for 60 sec. followed by another 27 cycles with an increased annealing temperature at 56°C, with a final extension at 72°C for 10 minutes. The ~1.6 kb PCR product was purified using a QIAEX Gel Extraction Kit as described (Ex. 3).

The purified PCR fragment was then digested with *Bsp*EI. pAXBΔpolΔpTP was digested with *Bsp*EI followed by treatment with SAP. The *Bsp*EI-digested PCR fragment and the *Bsp*EI-SAP-treated pAXBΔpolΔpTP were ligated together to create pAXBΔpolΔpTPVARNA+tl3.

b) Construction Of pBHG11ΔpolΔpTPVARNA+tl3

pAXBΔpolΔpTPVARNA+tl3 contains a 2.3 kb deletion within the pol and pTP genes (bp 8.773 to 11.091: numbering relative to that of pBHG11) and lacks the 11.4 kb *Bam*HI fragment containing the right arm of the Ad genome.

To provide the right arm of the Ad genome. pAXBΔpolΔpTPVARNA+tl3 was digested with *Bam*HI followed by treatment with SAP. pBHG11ΔE4 (pBHG11 or pBHG10 may be used in place of pBHG11ΔE4) was digested with *Bam*HI and the 11.4 kb fragment was isolated and purified using a QIAEX Gel Extraction Kit as described (Ex. 3). The purified 11.4 kb *Bam*HI fragment was ligated to the *Bam*HI/phosphatased pAXBΔpolΔpTPVARNA+tl3 to generate pBHG11ΔpolΔpTPVARNA+tl3. Proper construction of pBHG11ΔpolΔpTPVARNA+tl3 was confirmed restriction digestion.

c) Rescue And Propagation Of Ad Vectors Containing The pol, pTP Double Deletion

The Ad genome contained within pBHG11ΔpolΔpTPVARNA+tl3 lacks the Ad packaging signals. Therefore. in order to recover virus containing the 2.3 kb deletion within the pol and pTP genes from pBHG11ΔpolΔpTPVARNA+tl3. this plasmid must be cotransfected into packaging cells along with DNA that provides a source of the Ad packaging signals. The Ad packaging signals may be provided by wild-type or mutant Ad viral DNA or alternatively may be provided using a shuttle vector which contains the left-end

Ad5 sequences including the packaging signals such as pΔE1sp1A. pΔE1sp1B (Microbix) or pAdBglII.

Recombinant vAd vectors containing the pol. pTP double deletion which also contain a deletion within the E3 region are generated by cotransfection of

5   pBHG11ΔpolΔpTPVARNA+tl3 (the gene of interest is inserted into the unique *Pac*l site of pBHG11ΔpolΔpTPVARNA+tl3) with a E3-deleted Ad virus such as Ad5*dl*7001 into pol- and pTP-expressing 293 cells (*e.g.*. C-7); viral DNA-TPC are utilized as described (Ex. 3).

Recombinant vectors containing the pol. pTP double deletion which also contain deletions within the E1 and E3 regions are generated by cotransfection of

10   pBHG11ΔpolΔpTPVARNA+tl3 into pol- and pTP-expressing 293 cells (*e.g.*. C-7) along with a shuttle vector containing the left-end Ad5 sequences including the packaging signals. Suitable shuttle vectors include pΔE1sp1A (Microbix), pΔE1sp1B (Microbix) or pAdBglII. The gene of interest is inserted into the polylinker region of the shuttle vector and this plasmid is then cotransfected into B-6 or C-7 cells along with

15   pBHG11ΔpolΔpTPVARNA+tl3 to generate a recombinant E1⁻. pol ⁻. pTP⁻ Ad vector containing the gene of interest.

EXAMPLE 6

Encapsidated Adenovirus Minichromosomes
20   Containing A Full Length Dystrophin cDNA

In this Example. the construction of an encapsidated adenovirus minichromosome (EAM) consisting of an infectious encapsidated linear genome containing Ad origins of replication. packaging signal elements. a β-galactosidase reporter gene cassette and a full

25   length (14 kb) dystrophin cDNA regulated by a muscle specific enhancer/promoter is described. EAMs are generated by cotransfecting 293 cells with supercoiled plasmid DNA (pAd5βdys) containing an embedded inverted origin of replication (and the remaining above elements) together with linear DNA from E1-deleted virions expressing human placental alkaline phosphatase (hpAP). All proteins necessary for the generation of EAMs are provided

30   *in trans* from the hpAP virions and the two can be separated from each other on equilibrium CsCl gradients. These EAMs are useful for gene transfer to a variety of cell types both *in vitro* and *in vivo*.

a)   **Generation And Propagation Of Encapsidated Adenovirus Minichromosomes**

To establish a vector system capable of delivering full length dystrophin cDNA clones, the minimal region of Ad5 needed for replication and packaging was combined with a conventional plasmid carrying both dystrophin and a β-galactosidase reporter gene. In the first vector constructed, these elements were arranged such that the viral ITRs flanked (ITRs facing outward) the reporter gene and the dystrophin gene [*i.e.*, the vector contained from 5' to 3' the right or 3' ITR (mu 100 to 99), the dystrophin gene and the reporter gene and the left or 5' ITR and packaging sequnce (mu 1 to 0)]. Upon introduction of this vector along with helper virus into 293 cells, no encapsidated adenovirus minichromosomes were recovered.

The second and successful vector, pAd5βdys (Fig. 10) contains 2.1 kb of adenovirus DNA, together with a 14 kb murine dystrophin cDNA under the control of the mouse muscle creatine kinase enhancer/promoter, as well as a β-galactosidase gene regulated by the human cytomegalovirus enhancer/promoter.

Figure 10 shows the structure of pAd5βdys (27.8 kb). The two inverted adenovirus origins of replication are represented by a left and right inverted terminal repeat (LITR/RITR). Replication from these ITRs generates a linear genome whose termini correspond to the 0 and 100 map unit (mu) locations. Orientation of the origin with respect to wild type adenovirus serotype 5 sequences in mu is indicated above the figure (1 mu=360 bp). Encapsidation of the mature linear genome is enabled by five (AI-AV) packaging signals (Ψ). The *E. coli* β-galactosidase and *mus musculus* dystrophin cDNAs are regulated by cytomegalovirus (CMV) and muscle creatine kinase (MCK) enhancer/promoter elements, respectively. Both expression cassettes contain the SV40 polyadenylation (pA) signal. Since the E1A enhancer/promoter overlaps with the packaging signals, pAd5βdys was engineered such that RNA polymerase transcribing from the E1A enhancer/promoter will encounter the SV40 late polyadenylation signal. Pertinent restriction sites used in constructing pAd5βdys are indicated below the figure. An adenovirus fragment corresponding to mu 6.97 to 7.77 was introduced into pAd5βdys during the cloning procedure (described below). P1 and P2 represent location of probes used for Southern blot analysis. Restriction sites destroyed during the cloning of the Ad5 origin of replication and packaging signal are indicated in parentheses.

pAd5βdys was constructed as follows. pBSX (Ex. 3a) was used as the backbone for construction of pAd5βdys. The inverted fused Ad5 origin of replication and five encapsidation signals were excised as a *PstI/XbaI* fragment from pAd5ori, a plasmid containing the 6 kb *Hind*III fragment from pFG140 (Microbix Biosystems). This strategy also introduced a 290 bp fragment from Ad5 corresponding to map units 6.97 to 7.77 adjacent to the right inverted repeat (see Fig. 10). The *E. coli* β-galactosidase gene regulated by the human CMV immediate early (IE) promoter/enhancer expression cassette was derived as an *EcoRI/Hind*III fragment from pCMVβ [MacGregor and Caskey (1989) Nucleic Acids Res. 17:2365]: the CMV IE enhancer/promoter is available from a number of suppliers as is the *E. coli* β-galactosidase gene]. The murine dystrophin expression cassette was derived as a *BssH*II fragment from pCVAA, and contains a 3.3 kb MCK promoter/enhancer element [Phelps *et al.* (1995) Hum. Mol. Genet. 4:1251 and Jaynes *et al.* (1986) Mol. Cell. Biol. 6:2855]. The sequence of the ~3.3 kb MCK promoter/enhancer element is provided in SEQ ID NO:9 (in SEQ ID NO:9, the last nucleotide of SEQ ID NO:9 corresponds to nucleotide +7 of the MCK gene). The enhancer element is contained within a 206 bp fragment located -1256 and -1050 upstream of the transcription start site in the MCK gene.

It was hoped that the pAd5βdys plasmid would be packageable into an encapsidated minichromosome when grown in parallel with an E1-deleted virus due to the inclusion of both inverted terminal repeats (ITR) and the major Ad packaging signals in the plasmid. The ITRs and packaging signals were derived from pFG140 (Microbix), a plasmid that generates E1-defective Ad particles upon transfection of human 293 cells.

hpAP is an E1 deleted Ad5 containing the human placental alkaline phosphatase gene [Muller *et al.* (1994) Circ. Res. 75:1039]. This virus was chosen to provide the helper functions so that it would be possible to monitor the titer of the helper virus throughout serial passages by quantitative alkaline phosphatase assays.

293 cells were cotransfected with pAd5βdys and hpAP DNA as follows. Low passage 293 cells (Microbix Biosystems) were grown and passaged as suggested by the supplier. Five pAd5βdys and hpAP DNA (5 and 0.5 µg, respectively) were dissolved in 70 µl of 20 mM HEPES buffer (pH 7.4) and incubated with 30 µl of DOTAP (BMB) for 15 min. at room temperature. This mixture was resuspended in 2 mls of DMEM supplemented with 2% fetal calf serum (FCS) and added dropwise to a 60 mm plate of 293 cells at 80% confluency. Four hours post-transfection the media was replaced by DMEM with 10% FCS. Cytopathic effect was observed 6-12 days post-transfection.

Cotransfection of 293 cells with supercoiled pAd5βdys and linear hpAP DNA produced Ad5βdys EAMs (the encapsidated version of pAd5βdys) approximately 6 to 12 days post transfection. as evidenced by the appearance of a cytopathic effect (CPE). Initially. the amount of Ad5βdys EAMs produced was significantly lower than that of hpAP virions so the viral suspension was used to re-infect fresh cultures. from which virus was isolated and used for serial infection of additional cultures (Fig. 11). Infection and serial passaging of 293 cells was carried out as follows.

Lysate from one 60 mm plate of transfected 293 cells was prepared by vigorously washing the cells from the plate and centrifuging at 1 K rpm in a clinical centrifuge. Cells were resuspended in DMEM and 2% FCS. freeze-thawed in a dry ice-ethanol bath. cell debris removed by centrifugation. and approximately 75% of the crude lysate was used to infect 293 cells in DMEM supplemented with 2% FCS for 1 hour and then supplemented with 10% FCS thereafter. Infection was allowed to proceed for 18-20 hrs before harvesting the virus. The total number of cells infected in each serial passage is indicated in Figure 11.

In Figure 11. the total number of transducing adenovirus particles produced (output) per serial passage on 293 cells. total input virus of either the helper (hpAP) or Ad5βdys. and the total number of cells used in each infection is presented. The total number of input/output transducing particles were determined by infection of 293 cells plated in 6-well microtiter plates. Twenty four hours post-infection the cells were assayed for alkaline phosphatase or β-galactosidase activity (described below) to determine the number of cells transduced with either Ad5βdys or hpAP. The number of transducing particles were estimated by extrapolation of the mean calculated from 31 randomly chosen 2.5 mm^2 sectors of a 961 mm^2 plate. The intra-sector differences in total output of each type of virus are presented as the standard deviations. σ. in Figure 11. For each serial passage. 75% of the total output virus from the previous passage was used for infection.

Alkaline phosphatase or β-galactosidase activity was determined as follows. For detection of alkaline phosphatase. infected 293 cells on Petri dishes were rinsed twice with phosphate buffered saline (PBS) and fixed for 10 minutes in 0.5% glutaraldehyde in PBS. Cells were again rinsed twice with PBS for ten minutes followed by inactivation of endogenous alkaline phosphatase activity at 65°C for 1 hr. in PBS prior to the addition of the chromogenic substrate BCIP (5-bromo-4-chloro-3-indolyl phosphate) at 0.15 mg/ml and nitro blue tetrazolium at 0.3 mg/ml) . Cells were incubated at 37°C in darkness for 3- 24 hrs. For β-galactosidase assays. the cells were fixed and washed as above. then assayed using standard

methods [MacGregor et al. (1991) In Murray (ed.), *Methods in Molecular Biology. Vol. ͡ Gene Transfer and Expression Protocols*, Human Press Inc., Clifton, NJ. pp. 217-225].

As shown in Figure 11, the rate of increase in titer of Ad5βdys EAMs between transfection and serial passage 6 was approximately 100 times greater than that for hpAP virions. This result indicated that Ad5βdys has a replication advantage over the helper virus, probably due to the shorter genome length (a difference of approximately 8 kb) and hence an increased rate of packaging.

Interestingly, after serial passage 6 there was a rapid decrease in the total titer of hpAP virions whereas the titer of Ad5βdys EAMs continued to rise. While not limiting the present invention to any particular mechanism, at least two possible mechanisms could be responsible for this observation. Firstly, a buildup of defective hpAP virions due to infections at high multiplicities may slowly out-compete their full length counterparts, a phenomenon that has been previously observed upon serial propagation of adenovirus [Daniell (1976) J. Virol. 19:685; Rosenwirth et al. (1974) Virology 60:431; and Burlingham et al. (1974) Virology 60:419]. Secondly, the emergence of replication competent virions due to recombination events between E1 sequences in cellular DNA and the hpAP genome could lead to a buildup of virus particles defective in expressing alkaline phosphatase [Lochmüller et al. (1994) Hum. Gene Ther. 5:1485].

Southern analysis of DNA prepared from serial lysates 3, 6, 9 and 12 indicated that full length dystrophin sequences were present in each of these lysates (Fig. 12A). In addition, the correct size restriction fragments were detected using both dystrophin and β-galactosidase probes against lysate DNA digested with several enzymes (Figs. 12A-B).

Figure 12 shows a Southern blot analysis of viral DNA from lysates 3, 6, 9 and 12, digested with the restriction enzymes BssHII, NruI and EcoRV, indicating the presence of a full length dystrophin cDNA in all lysates. Fragments from the C terminus of *mus musculus* dystrophin cDNA (A) or the N terminus of *E coli* β-galactosidase (B) were labeled with dCTP[32] and used as probes [Sambrook et al., *supra*]. The position of these probes and the predicted fragments for each digest is indicated in Figure 10. Note that one end of each fragment (except the 17.8 kb BssHII dystrophin fragment) detected is derived from the end of the linearized Ad5βdys genome (see Fig. 10). Low levels of shorter products, presumably derived from defective virions, become detectable only at high serial passage number.

At the later passages (9 and 12) there appeared to be an emergence of truncated Ad5βdys sequences, suggesting that deletions and/or rearrangements may be occurring at later

passages. Hence. most experiments were performed with Ad5βdys EAMs derived from the earlier passages. The possibility of the emergence of replication competent (E1-containing) viruses was also examined by infection of HeLa cells by purified and crude serial lysates. none of which produced any detectable CPE.

5

b)   Purification Of Encapsidated Adenovirus Minichromosomes On CsCl Gradients

The ability to separate Ad5βdys EAMs from hpAP virions based on their buoyancy difference (due presumably to their different genome lengths) on CsCl gradients was

10   examined. Repeated fractionation of the viral lysate allows small differences in buoyancy to be resolved. CsCl purification of encapsidated adenovirus minichromosomes was performed as follows. Approximately 25% of the lysate prepared from various passages during serial infections was used to purify virions. Freeze-thawed lysate was centrifuged to remove the cell debris. The cleared lysate was extracted twice with 1.1.2 tricholorotrifluoroethane

15   (Sigma) and applied to CsCl step and self forming gradients.

Purification of virus was initially achieved by passing it twice through CsCl step gradients with densities of ρ=1.45 and ρ=1.20 in a SW28 rotor (Beckman). After isolation of the major band in the lower gradient. the virus was passed through a self forming gradient (initial ρ=1.334) at 37.000 rpm for 24 hrs followed by a relaxation of the gradient by

20   reducing the speed to 10.000 rpm for 10 hrs. in a SW41 rotor (Beckman) at 12°C [Anet and Strayer (1969) Biochem. Biophys. Res. Commun. 34:328]. The upper band from the gradient (composed mainly of Ad5βdys virions) was isolated using an 18 gauge needle. reloaded on a fourth CsCl gradient (ρ=1.334) and purified at 37.000 rpm for 24 hrs followed by 10.000 rpm for 10 hrs at 12°C.

25   The Ad5βdys-containing CsCl band was removed in 100 μl fractions from the top of the centrifugation tube and CsCl was removed by chromatography on Sephadex G-50 (Pharmacia). Aliquots from each fraction were used to infect 293 cells followed by β-galactosidase and alkaline phosphatase assays to quantitate the level of contamination by hpAP virions in the final viral isolate.

30   Results of the physical separation between Ad5βdys EAMs and hpAP virions are shown in Figure 13. Figure 13 shows the physical separation of Ad5βdys from hpAP virions at the third (A) and final (B) stages of CsCl purification (initial ρ=1.334) in a SW41 tube. Aliquots of Ad5βdys EAMs from the final stage were drawn through the top of the

centrifugation tube and assayed for β-galactosidase and alkaline phosphatase expression. Results of these assays are presented in Figure 14.

In Figure 14, shows the level of contamination of Ad5βdys EAMs by hpAP virions obtained from passage 6. Following four cycles of CsCl purification, aliquots were removed from the top of the centrifugation tube and used for infection of 293 cells, which were fixed 24 hrs later and assayed for β-galactosidase or alkaline phosphatase activity (described below). The number of transducing particles are an underestimation of the actual totals as in some cases a positive cell may have been infected by more than one transducing particle. The ratio of the two types of virions - Ad5βdys EAMs (LacZ) or hpAP (AP) in each fraction is indicated in the lower graph.

The maximum ratio of transducing Ad5βdys EAMs to hpAP virions reproducibly achieved in this study was 24.8 -- a contamination with helper virus corresponding to approximately 4% of the final viral isolate.

c) **Dystrophin And β-galactosidase Expression By Encapsidated Adenovirus Minichromosomes In Muscle Cells**

To determine if the Ad5βdys EAMs were able to express β-galactosidase and dystrophin in muscle cells, mouse *mdx* myogenic cultures were infected with CsCl purified EAMs.

i) **Propagation And Infection Of Muscle Cells**

MM14 and *mdx* myogenic cell lines were kindly provided by S. Hauschka (University of Washington) and were cultured as previously described [Linkhart *et al.* (1981) Dev. Biol. 86:19 and Clegg et al. (1987) J. Cell Biol. 105:949]. Briefly, myoblasts were grown on plastic tissue culture plates coated with a 0.1% gelatin in Ham's F-10 medium containing 15% (v/v) horse serum, 0.8 mM $CaCl_2$, 200 ng/ml recombinant human basic fibroblast growh factor (b-FGF) and 60 μg/ml genitimicin (proliferation medium). Cutures were induced to differentiate by switching to growth in the presence of growth medium lacking b-FGF and containing 10% horse serum (differentiation medium). Myoblasts or differentiated myotubes (three days post switching) were infected at a multiplicity of infection of 2.2 Ad5βdys EAMs per cell. Fractions containing minimal contamination with hpAP virions (3, 4 and 5 of passage 6) were used for western and immunofluorescence analysis. Infection was allowed to proceed for 3 days for both the myoblasts and myotubes before harvesting cells.

ii)   Total Protein Extraction And Immunoblot
           Analysis

For protein extraction, muscle cells were briefly trypsinized, transferred to a microcentrifuge tube, centrifuged at 14 K for 3 min at room temp and resuspended two times in PBS. After an additional centrifugation, the cell pellet was resuspended in 80 µl of RIPA buffer (50 mM Tris-Cl, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS) (Sambrook *et al.*, *supra*). The sample was briefly sheared using a 22 gauge needle to reduce viscosity and total protein concentration assayed using the bicinconinic acid protein assay reagent (Pierce, Rockford, IL). Expression of full length dystrophin or β-galactosidase in infected *mdx* and MM14 myoblasts or myotubes was analyzed by electrophoresis of 40 µg of total protein extract on a 6% SDS-PAGE gel (in 25 mM Tris, 192 mM glycine, 10 mM β-mercaptoethanol, 0.1% SDS). After transferring to Gelman Biotrace NT membrane (in 25 mM Tris, 192 mM glycine, 10 mM β-mercaptoethanol, 0.05% SDS, 20% methanol), the membrane was blocked with 5% non-fat milk and 1% goat-serum in Tris-buffered saline-Tween (TBS-T) for 12 hrs at 4°C. Immunostaining was done according to the protocol for the ECL western blotting detection reagents (Amersham Life Sciences, Buckingham, UK). The primary antibodies used were Dys-2 (Vector Laboratories) and anti-β-galactosidase (BMB, Indianapolis, IN) with a horseradish peroxidase-conjugated anti-mouse secondary antibody.

Western blot analysis of EAM-infected *mdx* myoblasts and myotubes (three days post-fusion) indicated that EAMs were able to infect both of these cell types (Figure 15). In Figure 15, immunoblots of protein extracts from *mdx* myoblasts and myotubes demonstrating the expression of β-galactosidase (A) and dystrophin (B) in cells infected with Ad5βdys EAMs. Total protein was extracted 3 days post infection in all cases. Myotubes were infected at three days following a switch to differentiation media. In Figure 15A, lane 1 contains total protein extract from 293 cells infected with a virus expressing β-galactosidase as a control (RSV-LacZ); lanes 2-5 contain total protein extracts from uninfected *mdx* myoblasts, *mdx* myoblasts infected with Ad5βdys EAMs, *mdx* myotubes and *mdx* myotubes derived from mdx myoblasts infected with Ad5βdys EAMs, respectively. In Figure 15B, lanes 1 and 7 contain total protein from mouse muscle ("C57") while lane 2 contains protein from wild type MM14 myotubes, as controls. Lanes 3-5 contain total protein extracts from uninfected *mdx* myoblasts, *mdx* myoblasts infected with Ad5βdys EAMs, *mdx* myotubes and *mdx* myotubes derived from mdx myoblasts infected with Ad5βdys EAMs, respectively.

As shown in Figure 15. expression of β-galactosidase was detected in both the infected *mdx* myoblasts and myotubes indicating that the CMV promoter was active at both these early stages of differentiation in muscle cells. However. only infected *mdx* myotubes produced protein detected by dys-2. an antibody recognizing the 17 C-terminal amino acids of dystrophin (Fig. 15). No dystrophin expression was detected in infected myoblasts by western analysis. indicating that the muscle creatine kinase promoter functions minimally. if at all. within the Ad5βdys EAM prior to terminal differentiation of these cells.

Dystrophin expression in *mdx* cells infected with EAMs was confirmed by immunofluorescence studies using N-terminal dystrophin antibodies. In agreement with the western analysis. dystrophin expression from the MCK promoter was detected only in differentiated *mdx* myotubes infected by Ad5βdys EAMs (Fig. 16). Figures 16A-C show immunofluorescence of dystrophin in wild type MM14 myotubes (A). uninfected *mdx* (B) and infected *mdx* myotubes (C). respectively. The results shown in Figure 16 demonstrate the transfer and expression of recombinant dystrophin to differentiated *mdx* cells by Ad5βdys EAMs.

Immunofluorescence of myogenic cells was performed as follows. Approximately 1.5 x 10⁶ MM14 or *mdx* myoblasts were plated on Poly-L-lysine (Sigma) coated glass slides (7 x 3 cm) which had been previously etched with a 0.05% chromium potassium sulfate and 0.1% gelatin solution. For myotube analysis. the cultures were switched to differentiation media [Clegg *et al.* (1987). *supra*] 48 hours after plating. immediately infected and then allowed to fuse for 3 days. whereas myoblasts were continuously propagated in proliferation media [Clegg *et al.* (1987). *supra*]. Cells were washed three times with PBS at room temperature and fixed in 3.7% formaldehyde. For immunostaining. cells were incubated in 0.5% Triton X-100. blocked with 1% normal goat serum and incubated with an affinity purified antibody against the N-terminus of murine dystrophin for 2 hrs. followed by extensive washing in PBS and 0.1% Tween-20 with gentle shaking. Cells were incubated with a 1:200 dilution of biotin conjugated anti-rabbit antibody (Pierce) for one hour and washed as above. Cells were further incubated with a 1:300 dilution of streptavidin-fluorescein isothiocyanate conjugate (Vectorlabs. Burlingam. CA) for one hour and washed as above. followed by extensive washing in PBS.

The above results show that embedded inverted Ad origins of replication coupled to an encapsidation signal can convert circular DNA molecules to linear forms in the presence of helper virus and that these genomes can be efficiently encapsidated and propagated to high

titers. Such viruses can be purified on a CsCl gradient and maintain their ability to transduce cells *in vitro.* and their increased cloning capacity allows the inclusion of large genes and tissue specific gene regulatory elements. The above results also show that the dystrophin gene was expressed in cells transduced by such viruses and that the protein product was correctly localized to the cell membrane. The above method for preparing EAMs theoretically enables virtually any gene of interest to be inserted into an infectious minichromosome by conventional cloning in plasmid vectors, followed by cotransfection with helper viral DNA in 293 cells. This approach is useful for a variety of gene transfer studies *in vitro.* The observation that vectors completely lacking viral genes can be used to transfer a full-length dystrophin cDNA into myogenic cells indicates that this method may be used for the treatment of DMD using gene therapy.

d)   A Modified MCK Enhancer Increases Expression Of Linked Genes In Muscle

The DNA fragment containing enhancer/promoter of the MCK gene utilized in the Ad5βdys EAM plasmid is quite large (~3.3. kb). In order to provide a smaller DNA fragment capable of directing high levels of expression of linked genes in muscle cells, portions of the 3.3 kb MCK enhancer/promoter were deleted and/or modified and inserted in front of a reporter gene (*lacZ*). The enhancer element of the MCK gene was modified to produce the 2RS5 enhancer: the sequence of the 2RS5 enhancer is provided in SEQ ID NO:10. The first 6 residues of SEQ ID NO:10 represent a *Kpn*I site added for ease of manipulation of the modified MCK enhancer element. Residue number 7 of SEQ ID NO:10 corresponds to residue number 2164 of the wild-type MCK enhancer sequence listed in SEQ ID NO:9 (position 2164 of SEQ ID NO:9 corresponds to position -1256 of the MCK gene). Residue number 174 of SEQ ID NO:10 corresponds to residue number 2266 of the wild-type MCK enhancer sequence listed in SEQ ID NO:9.

These MCK/lacZ constructs were used to transfect cells in culture (*i.e.,* myogenic cultures) or were injected as naked DNA into the muscle of mice and β-galactosidase activity was measured. Figure 17 provides a schematic of the MCK/lacZ constructs tested. The first construct shown in Figure 17 contains the 3.3. kb wild-type MCK enhancer/promoter fragment linked to the *E. coli lacZ* gene. The wild-type enhancer element (-1256 to -1056) is depicted by the box containing "E": the core promoter element (-358 to -80) is indicated by the light cross-hatching and the minimal promoter element (-80 to +7) is indicated by the dark

cross-hatching. The core promoter element is required in addition to the minimal promoter element (which is required for basal expression) in order to achieve increased muscle-specific expression in tissue culture [Shield *et al.* (1996) Mol. Cell. Biol. 16:5058]. The modified enhancer element. the 2RS5 enhancer. is indicated by the box labeled "E*." The box labeled "mi nx" contains a synthetic intron derived from adenovirus and is used to increase expression of the constructs (any intron may be utilized for this purpose). The box labeled "CMV" depicts the CMV IE enhancer/promoter which was used a positive control.

In figure 17. β-galactosidase activity is expressed relative to the activity of the wild-type MCK enhancer/promoter construct shown at the top of the figure. As shown in Figure 17. a construct containing the 2RS5 enhancer (SEQ ID NO:10) linked to either the minimal MCK promoter (a ~ 261 bp element) or the core and minimal MCK promoter elements (a ~539 bp element) directs higher levels of expression of the reporter gene in muscle cells as compared to the ~3.3 kb fragment containing the wild-type enhancer element. These modified enhancer/promoter elements are considerably smaller than the ~3.3 kb fragment used in the Ad5βdys EAM plasmid and are useful for directing the expression of foreign genes in muscle cells. These smaller elements are particularly useful for driving the expression of genes in the context of self-propagating adenoviral vectors which have more severe constraints on the amount of foreign DNA which can be inserted in comparison to the used of "gutted" adenoviruses such as the EAMs described above.

EXAMPLE 7

Generation Of High Titer Stocks Of Encapsidated Adenovirus
Minichromosomes Containing Minimal Helper Virus Contamination

The results presented in Example 6 demonstrated that encapsidated adenovirus minichromosomes (EAMs) can be prepared that lack all viral genes and which can express full-length dystrophin cDNAs in a muscle specific manner. The propagation of these EAMs requires the presence of helper adenoviruses that contaminate the final EAM preparation with conventional adenoviruses (about 4% of the total preparation). In this example the EAM system is modified to enable the generation of high titer stocks of EAMs with minimal helper virus contamination. Preferably the EAM stocks contain helper virus representing less than 1%. preferably less than 0.1% and most preferably less than 0.01% of the final viral isolate. Purified EAMs are then injected *in vivo* in muscles of dystrophin minus *mdx* mice to

determine whether these vectors lead to immune rejection and whether they can alleviate dystrophic symptoms in *mdx* muscles.

The amount of helper virus present in the EAM preparations is reduced in two ways. The first is by selectively controlling the relative packaging efficiency of the helper virus versus the EAM virus. The second is to improve physical methods for separating EAM from helper virus. These approaches enable the generation of dystrophin-expressing EAMs that are contaminated with minimal levels of helper virus.

a)   Development And Characterization Of Adenovirus Packaging Cell Lines Expressing The Cre Recombinase From Bacteriophage P1

Cell lines expressing a range of *Cre* levels are used to optimize the amount of helper virus packaging that occurs during growth of the EAM vectors. The *Cre-loxP* system [Sauer and Henderson (1988) Proc. Natl. Acad. Sci. USA 85:5166] is employed to selectively disable helper virus packaging during growth of EAMs. The bacterial *Cre* recombinase catalyzes efficient recombination between a 34 bp target sequence called *loxP*. To delete a desired sequence, *loxP* sites are placed at each end of the sequence to be deleted in the same orientation: in the presence of *Cre* recombinase the intervening DNA segment is efficiently excised.

Cell lines expressing a range of *Cre* recombinase levels are generated. The expression of too little *Cre* protein may result in high levels of helper virus being generated, which leads to unacceptably high levels of helper virus contaminating the final EAM preparation. If very high levels of *Cre* expression are present in a cell line, excision of the packaging signal from the helper virus would be 100% efficient (*i.e.*, it would completely prevent helper virus packaging). As shown in Example 6, serial passage of EAM preparations containing low levels of helper virus increased the titer of the EAM. Therefore, it is desirable, at least in the initial passages of a serial passage that some helper virus capable of being packaged is present. A low level of packagable helper virus may be provided by using a cell line expressing levels of *Cre* recombinase which are not high enough to achieve excision of the packaging signals from 100% of the helper virus: these cell lines would be used early in the serial passaging of the EAM stock and a cell line expressing high enough levels of *Cre* recombinanse to completely prevent helper virus packaging would be used for the final passage.

Alternatively. EAMs may be prepared using a packaging cell line that supports high efficiency *Cre* recombinase-mediated excision of the packaging signals from the helper virus by transfection of the *Cre*-expressing cell line with the EAM plasmid followed by infection of these cells with *loxP*-contaning helper virus using an MOI of ≥ 1.0.

Human 293 cell lines that express a variety of levels of *Cre* recombinase are generated as follows. An expression vector containing the *Cre* coding region. pOG231. was cotransfected into 293 cells along with pcDNA3 (Invitrogen); pcDNA3 contains the *neo* gene under the control of the SV40 promoter. pOG231 uses the human CMV enhancer/promoter [derived from CDM8 (Invitrogen)] to express a modified *Cre* gene [pOG231 was obtained from S. O'Gorman]. The modified *Cre* gene contained within pOG231 has had a nuclear localization signal inserted into the coding region to increase the efficiency of recombination.

pOG231 was constructed as follows. A *Bgl*II site was introduced into the 5' *Xba*I site of the synthethic intron of pMLSISCAT [Haung and Gorman (1990) Nucleic Acids Res. 18:937] by linker tailing. A *Bgl*II site in the synthethic intron of pMLSISCAT was destroyed and a *Bam*HI linker was inserted into the *Pst*I site at the 3' end of the synthethic intron in pMLSISCAT. *Bgl*II and *Sma*I sites and a nuclear localization signal were introduced into the 5' end of pMC-Cre [Gu *et al.* (1993) Cell 73:1155] using a PCR fragment that extended from the novel *Bgl*II and *Sma*I sites to the *Bam*HI site in the *Cre* coding region. This PCR fragment was ligated to a *Bam*HI/*Sal*I fragment containing a portion of the *Cre* coding region derived from pIC-Cre (Gu *et al.*. *supra*) and the intron plus Cre coding sequence was inserted into a modified form of pOG44 [O'Gorman *et al.* (1991) Science 251:1351] to generate pOG231. The predicted sequence of pOG231 from the *Bgl*II site to the *Bam*HI site located in the middle of the *Cre* coding sequence is listed in SEQ ID NO:11.

One 60 mm dish of 293 cells (Microbix) were transfected with 10 μg of *Pvu*II-linearized pOG231 and 1 μg of *Not*I-linearized pcDNA3 using a standard calcium phosphate precipitation protocol. Two days after the addition of DNA. the transfected cells were split into three 100 mm dishes and 1000 μg/ml of active G418 was added to the medium. The cells were fed periodically with G418-contaning medium and three weeks later. 24 G418-resistant clones were isolated.

The isolated clones were expanded for testing. Aliquots were frozen in liquid nitrogen at the earliest possible passage. The neomycin resistant cell lines were examined for the expression of *Cre* recombinase using following transfection assay. The neomycin resistant cells were transfected with PGK-1-GFP-lacZ (obtained from Sally Camper. Univ. of

Michigan. Ann Arbor. MI). which contains a green fluorescent protein (GFP) expression
cassette that can be excised by *Cre* recombinase to allow expression of β-galactosidase:
transfection was accomplised using standard calcium phosphate precipitation. Figure 18
provides a schematic of a GFP/β-gal reporter construct suitable for assaying the expression of
Cre recombinase in mammalian cells: GFP sequences and β-gal (*i.e., lacZ*) sequences are
avaialbale from commmerical sources (*e.g.,* Clonetech. Palo Alto. CA and Pharmacia Biotech.
Piscataway. NJ. respectively).

Control experiments verified that 293 cells transfected with PGK-1-GFP-lacZ
expressed significant amounts of β-galactosidase only if these cells also expressed *Cre*
recombinanse. β-galactosidase assays were performed as described in Example 6. Neomycin
resistant cells expressing *Cre* recombinase were grouped as high. medium or low expressors
based upon the amount of β-galactosidase activity produced (estimated by direct counting of
β-galactosidase-positive cells per high-power field and by observing the level of staining and
the rapidity with which the blue stain was apparent) when these cell lines were transfected
with the GFP/β-gal reporter construct. Thirteen positive (*i.e., Cre*-expressing) lines. D608#12.
#7. #22. #18. #17. #4. #8. #2. #2/2. #13. #5. #15 and #21 were retained for further use.

The results of this transfection analysis revealed that cultures of 293 cells expressing
medium to high levels of *Cre* recombinase could be generated without apparent toxicity.

b)   Generation Of Helper Adenovirus Strains That Contain *loxP*
Sites Flanking The Adenovirus Packaging Signals

Studies of EAM production demonstrated that the EAM vector has a packaging
advantage over the helper adenovirus (Ex. 6). While not limiting the present invention to a
particular mechanism. it is hypothesized that this packaging and replication advantage can be
greatly increased by using helper viruses that approach the packaging size limits of Ad5 [Bett
et al. (1993) J. Virol. 67:5911], by using viruses with mutations in E4 and/or E2 genes [Yeh
et al. (1996) J. Virol. 70:559: Gorziglia *et al.* (1996) J. Virol. 70:4173: and Amalfitano *et al.*
(1996) Proc. Natl. Acad. Sci. USA 93:3352]. by inclusion of mutations or alterations in the
packaging signals of the helper virus [Imler *et al.* (1995) Hum. Gene Ther. 6:711] and by
combining these strategies.

The *Cre-loxP* excision method is used to disable the packaging signals from the helper
virus genomes. The Ad5 packaging domain extends from nucleotide 194 to 358 and is
composed of five distinct elements that are functionally redundant [Hearing *et al.* (1987) J.

Virol. 61:2555]. Theoretically. any molecule containing the Ad5 origin of replication and packaging elements should replicate and be packaged into mature virions in the presence of non-defective helper virus. Disabling the packaging signals should allow replication and gene expression to proceed. but will prevent packaging of viral DNA into infectious particles. This in turn should allow the ratio of EAM to helper virus to be increased greatly.

To disable the packaging signals within the helper virus used to encapsidate the EAMs. the *loxP* sequences are incorporated into a helper virus that has a genome approaching the maximal packaging size for Ad [~105 map units: Bett *et al.* (1993). *supra*] which further decreases the efficiency of helper virus packaging. Final virus size can be adjusted by the choice of introns inserted into the reporter gene. by the choice of reporter genes. or by including a variety of DNA fragments of various sizes to act as "stuffer" fragments. A convenient reporter gene is the alkaline phosphatase gene (see Ex. 6). The optimized *Cre-loxP* system is also incorporated into a helper viral backbone containing disruptions of any or all of the E1-E4 genes. Use of such deleted genomes requires viral growth on appropriate complementing cell lines. such as 293 cells expressing E2. and/or E4 gene products. The *loxP* sequences are incorporated into the helper virus by placing the *loxP* sequences on either side of the packaging signals on a shuttle vector. This modified shuttle vector is then used to recombine with the Ad DNA derived from a virus containing disruptions of any or all of the E1-E4 genes to produce the desired helper virus containing the packaging signals flanked by *loxP* sequences.

pAdBglII. an adenovirus shuttle plasmid containing Ad sequences from 0-1 and 9-16 map units (mu) was used as the starting material. Synthetic oligonucleotides were used to create a polylinker which was inserted at 1 mu within pAdBglII as follows. The BglII LoxP oligo [5'-GAAGATCTATAACTTCGTATAATGTATGCTA TACGAAGTTATTACCGAAGAAATGGCTCGAGATCTTC-3' (SEQ ID NO:12) and its reverese complement [5'-GAAGATCTCGAGCCATTTCTTCGGTAATAACTTCGT ATAGCATACATTATACGAAGTTATAGATCTTC-3' (SEQ ID NO:13)] were synthesized. The AflIII LoxP oligo [5'-CCACATGTATAACTTCGTATAGCATACA TTATACGAAGTTATACATGTGG-3' (SEQ ID NO:14)] and its reverse complement [5'-CCACATGTATAACTTCGTATAATGTATGCTATACGAAGTTATACATG TGG-3' (SEQ ID NO:15)] were synthesized. The double stranded form of each *loxP* oligonuceotide was digested with the appropriate restriction enzyme (*e.g.*. *Bgl*II or *Afl*III) and inserted into pAdBglII which had been digested with *Bgl*II and *Afl*III. This resulted in the insertion of

LoxP sequences into the shuttle vector flanking the packaging signals that are located between 0.5 and 1 mu of Ad5 (one mu equals 360 bp in the Ad5 genome; the sequence of the Ad5 genome is lsited in SEQ ID NO:1). The 3' *loxP* sequence was inserted into the *Bg/*II site within pAdBglII. The 5' *loxP* sequence was inserted into the *Af/*III site located at base 143 (~0.8 mu) (numbering relative to Ad5).

DNA sequencing was used to verify the final structure of the modified shuttle plasmid between 0-1 mu. If *Cre* recombinase-mediated excision of the packaging signals is found to be too efficient (as judged by the production of too little helper virus), alternate sites for the insertion of the *loxP* sequences are used that would result in deletion of 2, 3 or 4 packaging sequences rather than all 5 [Grable and Hearing (1990) J. Virol. 64:2047]. The insertion of *loxP* sequences at sites along the Ad genome contained within the shuttle vector which would result in the deletion 2, 3 or 4 packaging sequences are easily made using the technique of recombinant PCR [Higuchi (1990) In: *PCR Protocols: A Guide to Methods and Applications*, Innis *et al.* (eds.) Academic Press, San Diego, CA, pp. 177-183]. The optimal amount of *Cre* recombinase-mediated excision of the packaging signals is that amount which permits the production of enough packaged helper virus to permit the slow spread of virus on the first plate of cells co-transfected with helper virus DNA (containing the *loxP* sequences) and the EAM vector. This permits serial passage of the EAM preparation onto a subsequent lawn of cells to increase the titer of the EAM preparation. Alternatively, if *Cre* recombinase-mediated excision of the packaging signals is essentially 100% efficient in all Cre-expressing cells lines (i.e., regardless of the level of *Cre*-expression, that is even a cell line expressing a low level of *Cre* as judged by the GFP/β-gal assay described above), the packaged EAMs may be used along with helper virus (used at a MOI of ~1.0) to infect the second or subsequent lawn of cells to permit serial passaging to increase the titer of the EAM preparation.

Following introduction of the *loxP* sequences into the shuttle vector, the human placental alkaline phosphatase (HpAp) cDNA under control of the RSV promoter was inserted into the polylinker to provide a reporter gene for the helper virus. This is the same reporter used previously during EAM generation (Ex. 6). The HpAp sequences were inserted as follows. The *loxP*-containing shuttle vector was linearized with *Xho*I and the hpAp cassette was ligated into the *Xho*I site (a *Xho*I site was inserted into pAdBglII during the insertion of the *loxP* sequences as a *Xho*I site was located on the 3' end of the *loxP* sequences inserted into the *Bg/*II site of pAdBglII). The HpAp cassette was constructed as follows.

pRSVhAPT40 (obtained from Gary Nabel. Univ. of Michigan. Ann Arbor. MI) was digested with EcoRI to generate an EcoRI fragment containing the HpAP cDNA and the SV40 intron and polyadenylation sequences. pRc/RSV (Invitrogen) was digested with HindIII. then partially digested with EcoRI. A 5.208 bp fragment was then size selected on an agarose gel.

5   treated with calf alkaline phosphatase. and ligated to the EcoRI fragment derived from pRSVhAPT40 to generate pRc/RSVAP. pRc/RSVAP was then digested with SaII and XhoI to liberate the RSV promoter linked to the HpAP cDNA cassette (including the SV40 intron and polyadenylation sequences). This SaII-XhoI fragment was inserted into the loxP-containing shuttle vector which had been digested with SaII and XhoI to generate pADLoxP-RSVAP.

10   Helper virus containing loxP sites flanking the Ad packaging signals is generated by co-transfection of the LoxP shuttle plasmid (pADLoxP-RSVAP) and ClaI-digested Ad5dl7001 DNA into 293 cells [Graham and Prevec (1991) Manipulation of Adenovirus Vectors. In Methods in Molecular Biology. Vol. 7: Gene Transfer and Expression Protocols. Murray (ed.). Humana Press Inc.. Clifton. NJ. pp. 109-128]. Figure 19 provides a schematic

15   showing the recombination event between the loxP shuttle vector and the Ad5dl7001 genome. Co-transfection is carried out as described in Example 3c.

Alternatively. the reporter gene may be inserted into the E3 region of pBHG10 or pBHG11 (using the unique PacI site) rather than into the polylinker located in the E1 region of the shuttle vector. The reporter gene-containing pBHG10 or 11 is then used in place of

20   Ad5dl7001 for cotransfection of 293 cells along with the loxP-containing pAdBglII.

Following cotransfection. recombinant plaques are picked. plaque purified. and tested for incorporation of both hpAp and loxP sequences by PCR and Southern analysis (Ex. 3). Viruses which contain loxP sites flanking the packaging signals and the marker gene (hpAp) are retained. propagated and purified.

25   The isolated helper virus containing loxP sites flanking the packaging signals and the marker gene is then used to infect both 293 cells and the 293 cell lines that express Cre (section a. above). Cre recombinase-expressing cell lines that produce optimal levels of Cre recombinase when infected with the loxP-containing helper virus are then used for the generation of EAMs as described below.

30

c)   Generation Of Encapsidated Adenovirus Minichromosomes
That Express Dystrophin

The growth of EAMs is optimized using the following methods. In the first method, plasmid DNA from the EAM vector (Ex. 6) are co-transfected into 293 cells with purified viral DNA from the helper virus, and viruses are harvested 10-14 days later after appearance of a viral cytopathic effect (CPE) as described in Example 6. This approach is simple, yet potentially increases helper virus levels by allowing viral spread throughout the culture dishes.

In the second method, the co-transfected 293 cells are overlaid with agar and single plaques are picked and tested for EAM activity by the ability to express β-galactosidase activity following infection of Hela cells (Ex. 6). This second approach is more time consuming, but will result in less contamination by helper.

The ability of these two methods to generate EAMs are directly compared. The preferred method is that which produces the highest titer of EAM with the lowest contamination of helper virus. The efficiency of EAM generation may also be increased by using helper virus DNA that retains the terminal protein (TP) (i.e., the helper virus is used as a viral DNA-TPC as described in Ex. 3) The use of viral DNA-TPC has been shown to increase the efficiency of viral production following transfection of 293 cells by an order of magnitude [Miyake et al. (1996), supra].

The initial transfection will utilize 293 cells that do not express Cre recombinase, so that efficient spread of the helper can lead to large scale production of EAM. The method producing the highest ratio of EAM to helper virus is then employed to optimize conditions for the serial propagation of the EAM on 293 cells expressing Cre recombinase. This optimization is conducted using cell lines expressing different levels of Cre recombinase. The following variables are tested: 1) the ratio of input viral titer to cell number, 2) the number of serial passages to use for EAM generation, 3) the use of cell lines producing different levels of Cre to achieve the optimal ratio between high EAM titer and low helper titer, 4) continuous growth on Cre-producing 293 cells versus alternating between Cre-producing cells and the parental 293 cells, or to alternate between high and low Cre-producing cells and 5) CsCl purification of EAMs prior to re-infection of 293 cells increases the ratio of the final EAM/helper titers. The protocols that result in the highest yield of EAM with minimal helper virus are used to generate large volumes of crude viral lysates for purification by density gradient centrifugation (Ex. 6).

EAMs were purified from helper virus using standard CsCl density gradient centrifugation protocols in Example 6 and resulted in preparations containing ~4% helper virus. In order to improve the physical separation of EAMs and helper virus, a variety of different centrifugation conditions are possible, including changing the gradient shape, type of rotor and tube used, combinations of step and continuous gradients, and the number of gradients used. Materials with better resolving powers than CsCl can also be employed. These include rubidium chloride and potassium bromide [Reich and Zarybnicky (1979) Annal. Biochem. 94:193].

d)   Use Of EAMs Encoding Dystrophin For Long Term Expression Of Dystrophin in Muscle

To demonstrate the ability of the purified dystrophin expressing EAMs (prepared as described above) to deliver and express dystrophin in the muscle of an animal, the following experiment is conducted. First, purified dystrophin-EAMs are delivered to the muscle by direct intramuscular injection into newborn, 1 month, and adult (3 month) mouse quadriceps muscle. The dystrophin expressing EAM Ad5βDys are injected into *mdx* mice and into transgenic *mdx* mice that express β-galactosidase in the pituitary gland [Tripathy *et al.* (1996) Nature Med. 2:545]. These latter mice are used to avoid potential immune-rejection of cells expressing β-galactosidase from the Ad5βDys vector. An alternate EAM lacking the β-galactosidase reporter gene may also be employed; however, the presence of the β-Gal reporter simplifies EAM growth and purification (vectors lacking β-Gal have their purity estimated by PCR assays rather than by β-Gal assays).

Following intramuscular injection of EAM, animals are sacrificed at intervals between 1 week and 6 months to measure dystrophin expression [by western blot analysis and by immunofluorescence (Phelps *et al.* (1995) Hum. Mol. Genet. 4:1251 and Rafael *et al.* (1996) J. Cell Biol. 134:93] and muscle extracts will also be assayed for β-Gal activity [MacGregor *et al.* (1991), *supra*]. These results are compared with previous results obtained using current generation viral vectors (*i.e.*, containing deletions in E1 and E3 only) to demonstrate that EAMs improve the prospects for long term gene expression in muscle.

e)   Use Of Dystrophin-EAMs To Prevent, Halt, Or Reverse The
         Dystrophic Symptoms That Develop In The Muscles Of *mdx*
         Mice

To demonstrate that beneficial effects on dystrophic muscle are achieved by delivery
of dystrophin expressing EAMs to *mdx* mice, the following experiments are conducted.
Central nuclei counts are performed on soleus muscle at intervals following injection of EAM
into newborn, 1 month, and 3 month (adult) mice (Phelps *et al.*, 1995). Central nuclei arise
in mouse muscle only after a myofiber has undergone dystrophic necrosis followed by
regeneration, and is a quantitative measure of the degree of dystrophy that has occurred in a
muscle group (Phelps *et al.*, 1995).

More informative assays are also contemplated. These assays require administration of
EAMs to mouse diaphragm muscles.

The diaphragm is severely affected in *mdx* mice (Stedman *et al.*, 1991; Cox *et al.*,
1993), and displays dramatic decreases in both force and power generation. Administration of
virus to the diaphragm will allow the strength of the muscles to be measured at intervals
following dystrophin delivery. The force and power generating assays developed by the
Faulkner lab are used to measure the effect of dystrophin transgenes (Shrager *et al.*, 1992;
Rafael *et al.*, 1994; Lynch *et al.*, 1996).

To detemine whether dystrophin delivery to dystrophic muscle reverses dystrophy or
stabilizes muscle, varying amounts of the dystrophin EAM are delivered to the to diaphragm
at different stages of the dystrophic process and then strength is measured at intervals
following EAM administration. First, various titers of EAM are tested to determine the
minimal amount of virus needed to transduce the majority of muscle fibers in the diaphragm.
It has been shown that conventional adenovirus vectors can transduce the majority of
diaphragm fibers when 10^8 pfu are administrated by direct injection into the intraperitoneal
cavity [Huard *et al.* (1995) Gene Therapy 2:107]. In addition, it has been shown that
transduction of a simple majority of fibers in a muscle group is sufficient to prevent virtually
all the dystrophic symptoms in mice [Rafael *et al.* (1994) Hum. Mol. Genet. 3:1725 and
Phelps *et al.* (1995) Hum. Mol. Genet. 4:1251]. Virus is administered to *mdx* animals at three
different ages (neonatal, 1 month, and 3 months). Animals are sacrificed for physiological
analysis of diaphragm muscle at two different times post infection (1 month and 3 months).
Error control is achieved by performing these experiments in sextuplicate. Control animals
consist of mock injected wild-type (C57Bl/10) and dystrophic (*mdx*) mice. Three month old

mdx mice display a 40% reduction in force and power generation compared with wild-type mice. while 6 month animals display a greater than 50% reduction (Cox *et al.* (1993) Nature 364:725; Rafael *et al.* (1994). *supra*; Phelps *et al.* (1995). *supra*; and Corrado *et al.* (1996) J. Cell. Biol. 134:873].

5

EXAMPLE 8

Improved Shuttle Vectors For The

Production Of Helper Virus Containing *LoxP* Sites

10   In the previous example. a shuttle vector containing adenoviral sequences extending from 9 mu to 16 mu of the Ad5 genome was modified to contain *loxP* sequences surrounding the packaging signals (the *LoxP* shuttle vector). This modified shuttle vector was then recombined with an Ad virus to produce a helper virus containing the *loxP* sequences. This helper virus was then used to infect cells expressing *Cre* recombinase along with DNA

15   comprising a minichromosome containing the dystrophin gene. a reporter gene and the packaging signals and ITRs of Ad in order to preferentially package the minichromosomes. Using this approach the helper virus. which has had the packaging signals removed by *Cre-loxP* recombination. contains the majority of the Ad genome (only a portion of the E3 region is deleted). Thus. if low levels of helper virus are packaged and appear in the EAM

20   preparation. the EAM preparation has the potential of passing on helper virus capable of directing the expression of Ad proteins in cells which are exposed to the EAM preparation. The expression of Ad proteins may lead to an immune response directed against the infected cells.

          Another approach to reducing the possibility that the EAM preparation contains helper

25   virus capable of provoking an immune response is to use helper viruses containing deletions and/or mutations within the pol and pTP genes. Helper virus containing a deletion in the pol and/or pTP genes is cotransfected with the EAM construct into 293-derived cell lines expressing pol or pol and pTP to produce EAMs. Any helper virus present in the purified EAM preparation will be replication defective due to the deletion in the pol and/or pTP genes.

30   As shown in Example 3. viruses containing a deletion in the pol gene are incapable of directing the expression of viral late genes: therefore. helper viruses containing a deletion in the pol gene or the pol and pTP genes should not be capable of provoking an immune response (*i.e.*. a CTL response) against late viral proteins synthesized *de novo*. Shuttle

vectors contain deletions within the Ad pol and/or pTP genes are constructed as described below.

a) Construction Of A Shuttle Vector Containing The Δpol Deletion

pAdBglII was modified to contain sequences corresponding to 9 to 40 mu of the Ad5 genome as follows. pAdBglII was digested with BglII and a linker/adapter containing an AscI site was added to create pAdBglIIAsc. pAdBglIIAsc was then digested with BstI1071. Ad5dl7001 viral DNA was digested with AscI and the ends were filled in using T4 DNA polymerase. The AscI-digested, T4 DNA polymerase filled Ad5dl7001 viral DNA was then digested with BstI1071 and the ~9.9 kb AscI-BstI1071(filled) fragment containing the pol and pTP genes was isolated (as described in Ex. 3) and ligated to the BstI1071-digested pAdBglIIAsc to generate pAdAsc. pAdAsc is a shuttle vector which contains the genes encoding the DNA polymerase and preterminal protein (the inserted AscI-BstI1071 fragment corresponds to nucleotides 5767 to 15, 671 of the Ad5 genome).

A shuttle vector, pAdAscΔpol, which contains the 612 bp deletion in the pol gene (described in Ex. 3) was constructed as follows. Ad5ΔpolΔE31 viral DNA (Ex. 3) was digested with AscI and the ends were filled in using T4 DNA polymerase. The AscI-digested, T4 DNA polymerase filled Ad5ΔpolΔE31 viral DNA was then digested with BstI1071 and the ~9.3 kb AscI-BstI1071(filled) fragment containing the deleted pol gene and the pTP gene was isolated (as described in Ex. 3) and ligated to BstI1071-digested pAdBglIIAsc to generate pAdAscΔpol.

b) Construction Of A Shuttle Vector Containing The ΔpolΔpTP Deletion

A shuttle vector, pAdAscΔpolΔpTP, which contains a 2.3 kb deletion within the pol and pTP genes (described in Ex. 5) was constructed as follows. pAXBΔpolΔpTPVARNA+tl3 (Ex. 5b) was digested with AscI and the ends were filled in using T4 DNA polymerase. The AscI-digested, T4 DNA polymerase filled pAXBΔpolΔpTPVARNA+tl3 DNA was then digested with BstI1071 and the ~7.6 kb AscI-BstI1071(filled) fragment containing the deleted pol gene and the pTP gene was isolated (as described in Ex. 3) and ligated to BstI1071-digested pAdBglIIAsc to generate pAdAscΔpolΔpTP.

In order to reduce the packaging of the above helper viruses, the pol⁻ or pol⁻, pTP⁻ helper viruses can be modified to incorporate loxP sequences on either side of the packaging

signals as outlined in Example 7. The *loxP*-containing pol⁻ or pol⁻. pTP⁻ shuttle vectors (pTP- shuttle vectors may also be employed) are cotransfected into 293 cells expressing *Cre* recombinase and pol or *Cre* recombinase. pol and pTP. respectively along with an appropriate E1⁻ viral DNA-TPC (the E1- viral DNA may also contain deletions elsewhere in the genome such as in the E4 genes or in the E2a gene as packaging cell lines expressing the E4 ORF 6 or 6/7 and lines expressing E2a genes are avaialble) to generate helper virus containing *loxP* sites flanking the packaging signals as well as a deletion in the pol gene or the pol and pTP genes. The resulting helper virus(es) is used to cotransfect 293 cells expressing pol or pol and pTP along with the desired EAM construct. The resulting EAM preparation should contain little if any helper virus and any contaminating helper virus present would be replication defective and incapable of expressing viral late gene products. Helper viruses containing *loxP* sequences and deletions in all essential early genes may be employed in conjunction with *Cre* recombinanse-expressing cell lines expressing *in trans* the E1. E4 ORF 6. E2a. and E2b (*e.g.*. Ad polymerase and pTP) proteins (the E3 proteins are dispensible for growth in culture). Cell lines coexpressing E1. polymerase and pTP are provided herein. Cell lines expressing E1 and E4 proteins have been recently described [Krougliak and Graham (1995) Hum. Gene Ther. 6:1575 and Wang *et al.* (1995) Gene Ther. 2:775] and cell lines expressing E1 and E2a proteins have been recently described [Zhou *et al.* (1996) J. Virol. 70:7030]. Therefore. a cell line co-expressing E1. E2a. E2b. and E4 is consructed by introduction of expression plasmids containing the E2a and E4 coding regions into the E1-. Ad polymerase- and pTP-expressing cell lines of the present invention. These packaging cell lines are used in conjunction with helper viruses containing deletions in the E1. E2a. E2b and E4 regions.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments. it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed. various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Chamberlain, Jeffrey S.
               Amalfitano, Andrea
               Hauser, Michael A.
               Kumar-Singh, Rajendra
               Hartigan-O'Connor, Dennis J.

    (ii) TITLE OF INVENTION: IMPROVED ADENOVIRUS VECTORS

    (iii) NUMBER OF SEQUENCES: 15

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Medlen & Carroll, LLP
        (B) STREET: 220 Montgomery Street, Suite 2200
        (C) CITY: San Francisco
        (D) STATE: California
        (E) COUNTRY: United States Of America
        (F) ZIP: 94104

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: US
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Ingolia, Diane E.
        (B) REGISTRATION NUMBER: 40,027
        (C) REFERENCE/DOCKET NUMBER: UM-02484

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (415) 705-8410
        (B) TELEFAX: (415) 397-8338

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35935 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "DNA"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT      60

TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT     120

GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGACGGATG TGGCAAAAGT GACGTTTTTG     180

GTGTGCGCCG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG     240

TAAATTTGGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG AATAAGAGGA     300

AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG     360

GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC     420

CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGCTGACG TGTAGTGTAT TTATACCCGG     480
```

```
TGAGTTCCTC AAGAGGCCAC TCTTGAGTGC CAGCGAGTAG AGTTTTCTCC TCCGAGCCGC       540

TCCGACACCG GGACTGAAAA TGAGACATAT TATCTGCCAC GGAGGTGTTA TTACCGAAGA       600

AATGGCCGCC AGTCTTTTGG ACCAGCTGAT CGAAGAGGTA CTGGCTGATA ATCTTCCACC       660

TCCTAGCCAT TTTGAACCAC CTACCCTTCA CGAACTGTAT GATTTAGACG TGACGGCCCC       720

CGAAGATCCC AACGAGGAGG CGGTTTCGCA GATTTTTCCC GACTCTGTAA TGTTGGCGGT       780

GCAGGAAGGG ATTGACTTAC TCACTTTTCC GCCGGCGCCC GGTTCTCCGG AGCCGCCTCA       840

CCTTTCCCGG CAGCCCGAGC AGCCGGAGCA GAGAGCCTTG GGTCCGGTTT CTATGCCAAA       900

CCTTGTACCG GAGGTGATCG ATCTTACCTG CCACGAGGCT GGCTTTCCAC CCAGTGACGA       960

CGAGGATGAA GAGGGTGAGG AGTTTGTGTT AGATTATGTG GAGCACCCCG GGCACGGTTG      1020

CAGGTCTTGT CATTATCACC GGAGGAATAC GGGGGACCCA GATATTATGT GTTCGCTTTG      1080

CTATATGAGG ACCTGTGGCA TGTTTGTCTA CAGTAAGTGA AAATTATGGG CAGTGGGTGA      1140

TAGAGTGGTG GGTTTGGTGT GGTAATTTTT TTTTTAATTT TTACAGTTTT GTGGTTTAAA      1200

GAATTTTGTA TTGTGATTTT TTTAAAAGGT CCTGTGTCTG AACCTGAGCC TGAGCCCGAG      1260

CCAGAACCGG AGCCTGCAAG ACCTACCCGC CGTCCTAAAA TGGCGCCTGC TATCCTGAGA      1320

CGCCCGACAT CACCTGTGTC TAGAGAATGC AATAGTAGTA CGGATAGCTG TGACTCCGGT      1380

CCTTCTAACA CACCTCCTGA GATACACCCG GTGGTCCCGC TGTGCCCCAT TAAACCAGTT      1440

GCCGTGAGAG TTGGTGGGCG TCGCCAGGCT GTGGAATGTA TCGAGGACTT GCTTAACGAG      1500

CCTGGGCAAC CTTTGGACTT GAGCTGTAAA CGCCCCAGGC CATAAGGTGT AAACCTGTGA      1560

TTGCGTGTGT GGTTAACGCC TTTGTTTGCT GAATGAGTTG ATGTAAGTTT AATAAAGGGT      1620

GAGATAATGT TTAACTTGCA TGGCGTGTTA AATGGGGCGG GGCTTAAAGG GTATATAATG      1680

CGCCGTGGGC TAATCTTGGT TACATCTGAC CTCATGGAGG CTTGGGAGTG TTTGGAAGAT      1740

TTTTCTGCTG TGCGTAACTT GCTGGAACAG AGCTCTAACA GTACCTCTTG GTTTTGGAGG      1800

TTTCTGTGGG GCTCATCCCA GGCAAAGTTA GTCTGCAGAA TTAAGGAGGA TTACAAGTGG      1860

GAATTTGAAG AGCTTTTGAA ATCCTGTGGT GAGCTGTTTG ATTCTTTGAA TCTGGGTCAC      1920

CAGGCGCTTT TCCAAGAGAA GGTCATCAAG ACTTTGGATT TTTCCACACC GGGGCGCGCT      1980

GCGGCTGCTG TTGCTTTTTT GAGTTTTATA AAGGATAAAT GGAGCGAAGA AACCCATCTG      2040

AGCGGGGGGT ACCTGCTGGA TTTTCTGGCC ATGCATCTGT GGAGAGCGGT TGTGAGACAC      2100

AAGAATCGCC TGCTACTGTT GTCTTCCGTC CGCCCGGCGA TAATACCGAC GGAGGAGCAG      2160

CAGCAGCAGC AGGAGGAAGC CAGGCGGCGG CGGCAGGAGC AGAGCCCATG GAACCCGAGA      2220

GCCGGCCTGG ACCCTCGGGA ATGAATGTTG TACAGGTGGC TGAACTGTAT CCAGAACTGA      2280

GACGCATTTT GACAATTACA GAGGATGGGC AGGGGCTAAA GGGGGTAAAG AGGGAGCGGG      2340

GGGCTTGTGA GGCTACAGAG GAGGCTAGGA ATCTAGCTTT TAGCTTAATG ACCAGACACC      2400

GTCCTGAGTG TATTACTTTT CAACAGATCA AGGATAATTG CGCTAATGAG CTTGATCTGC      2460

TGGCGCAGAA GTATTCCATA GAGCAGCTGA CCACTTACTG GCTGCAGCCA GGGGATGATT      2520

TTGAGGAGGC TATTAGGGTA TATGCAAAGG TGGCACTTAG GCCAGATTGC AAGTACAAGA      2580
```

```
TCAGCAAACT TGTAAATATC AGGAATTGTT GCTACATTTC TGGGAACGGG GCCGAGGTGG    2640

AGATAGATAC GGAGGATAGG GTGGCCTTTA GATGTAGCAT GATAAATATG TGGCCGGGGG    2700

TGCTTGGCAT GGACGGGGTG GTTATTATGA ATGTAAGGTT TACTGGCCCC AATTTTAGCG    2760

GTACGGTTTT CCTGGCCAAT ACCAACCTTA TCCTACACGG TGTAAGCTTC TATGGGTTTA    2820

ACAATACCTG TGTGGAAGCC TGGACCGATG TAAGGGTTCG GGGCTGTGCC TTTTACTGCT    2880

GCTGGAAGGG GGTGGTGTGT CGCCCCAAAA GCAGGGCTTC AATTAAGAAA TGCCTCTTTG    2940

AAAGGTGTAC CTTGGGTATC CTGTCTGAGG GTAACTCCAG GGTGCGCCAC AATGTGGCCT    3000

CCGACTGTGG TTGCTTCATG CTAGTGAAAA GCGTGGCTGT GATTAAGCAT AACATGGTAT    3060

GTGGCAACTG CGAGGACAGG GCCTCTCAGA TGCTGACCTG CTCGGACGGC AACTGTCACC    3120

TGCTGAAGAC CATTCACGTA GCCAGCCACT CTCGCAAGGC CTGGCCAGTG TTTGAGCATA    3180

ACATACTGAC CCGCTGTTCC TTGCATTTGG GTAACAGGAG GGGGGTGTTC CTACCTTACC    3240

AATGCAATTT GAGTCACACT AAGATATTGC TTGAGCCCGA GAGCATGTCC AAGGTGAACC    3300

TGAACGGGGT GTTTGACATG ACCATGAAGA TCTGGAAGGT GCTGAGGTAC GATGAGACCC    3360

GCACCAGGTG CAGACCCTGC GAGTGTGGCG GTAAACATAT TAGGAACCAG CCTGTGATGC    3420

TGGATGTGAC CGAGGAGCTG AGGCCCGATC ACTTGGTGCT GGCCTGCACC CGCGCTGAGT    3480

TTGGCTCTAG CGATGAAGAT ACAGATTGAG GTACTGAAAT GTGTGGGCGT GGCTTAAGGG    3540

TGGGAAAGAA TATATAAGGT GGGGGTCTTA TGTAGTTTTG TATCTGTTTT GCAGCAGCCG    3600

CCGCCGCCAT GAGCACCAAC TCGTTTGATG GAAGCATTGT GAGCTCATAT TTGACAACGC    3660

GCATGCCCCC ATGGGCCGGG GTGCGTCAGA ATGTGATGGG CTCCAGCATT GATGGTCGCC    3720

CCGTCCTGCC CGCAAACTCT ACTACCTTGA CCTACGAGAC CGTGTCTGGA ACGCCGTTGG    3780

AGACTGCAGC CTCCGCCGCC GCTTCAGCCG CTGCAGCCAC CGCCCGCGGG ATTGTGACTG    3840

ACTTTGCTTT CCTGAGCCCG CTTGCAAGCA GTGCAGCTTC CCGTTCATCC GCCCGCGATG    3900

ACAAGTTGAC GGCTCTTTTG GCACAATTGG ATTCTTTGAC CCGGGAACTT AATGTCGTTT    3960

CTCAGCAGCT GTTGGATCTG CGCCAGCAGG TTTCTGCCCT GAAGGCTTCC TCCCCTCCCA    4020

ATGCGGTTTA AAACATAAAT AAAAAACCAG ACTCTGTTTG GATTTGGATC AAGCAAGTGT    4080

CTTGCTGTCT TTATTTAGGG GTTTTGCGCG CGCGGTAGGC CCGGGACCAG CGGTCTCGGT    4140

CGTTGAGGGT CCTGTGTATT TTTTCCAGGA CGTGGTAAAG GTGACTCTGG ATGTTCAGAT    4200

ACATGGGCAT AAGCCCGTCT CTGGGGTGGA GGTAGCACCA CTGCAGAGCT TCATGCTGCG    4260

GGGTGGTGTT GTAGATGATC CAGTCGTAGC AGGAGCGCTG GGCGTGGTGC CTAAAAATGT    4320

CTTTCAGTAG CAAGCTGATT GCCAGGGGCA GGCCCTTGGT GTAAGTGTTT ACAAAGCGGT    4380

TAAGCTGGGA TGGGTGCATA CGTGGGGATA TGAGATGCAT CTTGGACTGT ATTTTTAGGT    4440

TGGCTATGTT CCCAGCCATA TCCCTCCGGG GATTCATGTT GTGCAGAACC ACCAGCACAG    4500

TGTATCCGGT GCACTTGGGA AATTTGTCAT GTAGCTTAGA AGGAAATGCG TGGAAGAACT    4560

TGGAGACGCC CTTGTGACCT CCAAGATTTT CCATGCATTC GTCCATAATG ATGGCAATGG    4620

GCCCACGGGC GGCGGCCTGG GCGAAGATAT TTCTGGGATC ACTAACGTCA TAGTTGTGTT    4680
```

```
CCAGGATGAG ATCGTCATAG GCCATTTTTA CAAAGCGCGG GCGGAGGGTG CCAGACTGCG      4740

GTATAATGGT TCCATCCGGC CCAGGGGCGT AGTTACCCTC ACAGATTTGC ATTTCCCACG      4800

CTTTGAGTTC AGATGGGGGG ATCATGTCTA CCTGCGGGGC GATGAAGAAA ACGGTTTCCG      4860

GGGTAGGGGA GATCAGCTGG GAAGAAAGCA GGTTCCTGAG CAGCTGCGAC TTACCGCAGC      4920

CGGTGGGCCC GTAAATCACA CCTATTACCG GGTGCAACTG GTAGTTAAGA GAGCTGCAGC      4980

TGCCGTCATC CCTGAGCAGG GGGGCCACTT CGTTAAGCAT GTCCCTGACT CGCATGTTTT      5040

CCCTGACCAA ATCCGCCAGA AGGCGCTCGC CGCCCAGCGA TAGCAGTTCT TGCAAGGAAG      5100

CAAAGTTTTT CAACGGTTTG AGACCGTCCG CCGTAGGCAT GCTTTTGAGC GTTTGACCAA      5160

GCAGTTCCAG GCGGTCCCAC AGCTCGGTCA CCTGCTCTAC GGCATCTCGA TCCAGCATAT      5220

CTCCTCGTTT CGCGGGTTGG GGCGGCTTTC GCTGTACGGC AGTAGTCGGT GCTCGTCCAG      5280

ACGGGCCAGG GTCATGTCTT TCCACGGGCG CAGGGTCCTC GTCAGCGTAG TCTGGGTCAC      5340

GGTGAAGGGG TGCGCTCCGG GCTGCGCGCT GGCCAGGGTG CGCTTGAGGC TGGTCCTGCT      5400

GGTGCTGAAG CGCTGCCGGT CTTCGCCCTG CGCGTCGGCC AGGTAGCATT TGACCATGGT      5460

GTCATAGTCC AGCCCCTCCG CGGCGTGGCC CTTGGCGCGC AGCTTGCCCT TGGAGGAGGC      5520

GCCGCACGAG GGGCAGTGCA GACTTTTGAG GGCGTAGAGC TTGGGCGCGA GAAATACCGA      5580

TTCCGGGGAG TAGGCATCCG CGCCGCAGGC CCCGCAGACG GTCTCGCATT CCACGAGCCA      5640

GGTGAGCTCT GGCCGTTCGG GGTCAAAAAC CAGGTTTCCC CCATGCTTTT TGATGCGTTT      5700

CTTACCTCTG GTTTCCATGA GCCGGTGTCC ACGCTCGGTG ACGAAAAGGC TGTCCGTGTC      5760

CCCGTATACA GACTTGAGAG GCCTGTCCTC GAGCGGTGTT CCGCGGTCCT CCTCGTATAG      5820

AAACTCGGAC CACTCTGAGA CAAAGGCTCG CGTCCAGGCC AGCACGAAGG AGGCTAAGTG      5880

GGAGGGGTAG CGGTCGTTGT CCACTAGGGG GTCCACTCGC TCCAGGGTGT GAAGACACAT      5940

GTCGCCCTCT TCGGCATCAA GGAAGGTGAT TGGTTTGTAG GTGTAGGCCA CGTGACCGGG      6000

TGTTCCTGAA GGGGGGCTAT AAAAGGGGGT GGGGGCGCGT TCGTCCTCAC TCTCTTCCGC      6060

ATCGCTGTCT GCGAGGGCCA GCTGTTGGGG TGAGTACTCC CTCTGAAAAG CGGGCATGAC      6120

TTCTGCGCTA AGATTGTCAG TTTCCAAAAA CGAGGAGGAT TTGATATTCA CCTGGCCCGC      6180

GGTGATGCCT TTGAGGGTGG CCGCATCCAT CTGGTCAGAA AAGACAATCT TTTTGTTGTC      6240

AAGCTTGGTG GCAAACGACC CGTAGAGGGC GTTGGACAGC AACTTGGCGA TGGAGCGCAG      6300

GGTTGGTTTT TTGTCGCGAT CGGCGCGCTC CTTGGCCGCG ATGTTTAGCT GCACGTATTC      6360

GCGCGCAACG CACCGCCATT CGGGAAAGAC GGTGGTGCGC TCGTCGGGCA CCAGGTGCAC      6420

GCGCCAACCG CGGTTGTGCA GGGTGACAAG GTCAACGCTG GTGGCTACCT CTCCGCGTAG      6480

GCGCTCGTTG GTCCAGCAGA GGCGGCCGCC CTTGCGCGAG CAGAATGGCG GTAGGGGGTC      6540

TAGCTGCGTC TCGTCCGGGG GGTCTGCGTC CACGGTAAAG ACCCCGGGCA GCAGGCGCGC      6600

GTCGAAGTAG TCTATCTTGC ATCCTTGCAA GTCTAGCGCC TGCTGCCATG CGCGGGCGGC      6660

AAGCGCGCGC TCGTATGGGT TGAGTGGGGG ACCCCATGGC ATGGGTGGG TGAGCGCGGA      6720

GGCGTACATG CCGCAAATGT CGTAAACGTA GAGGGGCTCT CTGAGTATTC CAAGATATGT      6780
```

- 72 -

```
AGGGTAGCAT CTTCCACCGC GGATGCTGGC GCGCACGTAA TCGTATAGTT CGTGCGAGGG    6840

AGCGAGGAGG TCGGGACCGA GGTTGCTACG GGCGGGCTGC TCTGCTCGGA AGACTATCTG    6900

CCTGAAGATG GCATGTGAGT TGGATGATAT GGTTGGACGC TGGAAGACGT TGAAGCTGGC    6960

GTCTGTGAGA CCTACCGCGT CACGCACGAA GGAGGCGTAG GAGTCGCGCA GCTTGTTGAC    7020

CAGCTCGGCG GTGACCTGCA CGTCTAGGGC GCAGTAGTCC AGGGTTTCCT TGATGATGTC    7080

ATACTTATCC TGTCCCTTTT TTTTCCACAG CTCGCGGTTG AGGACAAACT CTTCGCGGTC    7140

TTTCCAGTAC TCTTGGATCG GAAACCCGTC GGCCTCCGAA CGGTAAGAGC CTAGCATGTA    7200

GAACTGGTTG ACGGCCTGGT AGGCGCAGCA TCCCTTTTCT ACGGGTAGCG CGTATGCCTG    7260

CGCGGCCTTC CGGAGCGAGG TGTGGGTGAG CGCAAAGGTG TCCCTGACCA TGACTTTGAG    7320

GTACTGGTAT TTGAAGTCAG TGTCGTCGCA TCCGCCCTGC TCCCAGAGCA AAAAGTCCGT    7380

GCGCTTTTTG GAACGCGGAT TTGGCAGGGC GAAGGTGACA TCGTTGAAGA GTATCTTTCC    7440

CGCGCGAGGC ATAAAGTTGC GTGTGATGCG GAAGGGTCCC GGCACCTCGG AACGGTTGTT    7500

AATTACCTGG GCGGCGAGCA CGATCTCGTC AAAGCCGTTG ATGTTGTGGC CCACAATGTA    7560

AAGTTCCAAG AAGCGCGGGA TGCCCTTGAT GGAAGGCAAT TTTTTAAGTT CCTCGTAGGT    7620

GAGCTCTTCA GGGGAGCTGA GCCCGTGCTC TGAAAGGGCC CAGTCTGCAA GATGAGGGTT    7680

GGAAGCGACG AATGAGCTCC ACAGGTCACG GGCCATTAGC ATTTGCAGGT GGTCGCGAAA    7740

GGTCCTAAAC TGGCGACCTA TGGCCATTTT TTCTGGGGTG ATGCAGTAGA AGGTAAGCGG    7800

GTCTTGTTCC CAGCGGTCCC ATCCAAGGTT CGCGGCTAGG TCTCGCGCGG CAGTCACTAG    7860

AGGCTCATCT CCGCCGAACT TCATGACCAG CATGAAGGGC ACGAGCTGCT TCCCAAAGGC    7920

CCCCATCCAA GTATAGGTCT CTACATCGTA GGTGACAAAG AGACGCTCGG TGCGAGGATG    7980

CGAGCCGATC GGGAAGAACT GGATCTCCCG CCACCAATTG GAGGAGTGGC TATTGATGTG    8040

GTGAAAGTAG AAGTCCCTGC GACGGGCCGA ACACTCGTGC TGGCTTTTGT AAAAACGTGC    8100

GCAGTACTGG CAGCGGTGCA CGGGCTGTAC ATCCTGCACG AGGTTGACCT GACGACCGCG    8160

CACAAGGAAG CAGAGTGGGA ATTTGAGCCC CTCGCCTGGC GGGTTTGGCT GGTGGTCTTC    8220

TACTTCGGCT GCTTGTCCTT GACCGTCTGG CTGCTCGAGG GGAGTTACGG TGGATCGGAC    8280

CACCACGCCG CGCGAGCCCA AAGTCCAGAT GTCCGCGCGC GGCGGTCGGA GCTTGATGAC    8340

AACATCGCGC AGATGGGAGC TGTCCATGGT CTGGAGCTCC CGCGGCGTCA GGTCAGGCGG    8400

GAGCTCCTGC AGGTTTACCT CGCATAGACG GGTCAGGGCG CGGGCTAGAT CCAGGTGATA    8460

CCTAATTTCC AGGGGCTGGT TGGTGGCGGC GTCGATGGCT TGCAAGAGGC CGCATCCCCG    8520

CGGCGCGACT ACGGTACCGC GCGGCGGGCG GTGGGCCGCG GGGGTGTCCT TGGATGATGC    8580

ATCTAAAAGC GGTGACGCGG GCGAGCCCCC GGAGGTAGGG GGGGCTCCGG ACCCGCCGGG    8640

AGAGGGGGCA GGGGCACGTC GGCGCCGCGC GCGGGCAGGA GCTGGTGCTG CGCGCGTAGG    8700

TTGCTGGCGA ACGCGACGAC GCGGCGGTTG ATCTCCTGAA TCTGGCGCCT CTGCGTGAAG    8760

ACGACGGGCC CGGTGAGCTT GAGCCTGAAA GAGAGTTCGA CAGAATCAAT TTCGGTGTCG    8820

TTGACGGCGG CCTGGCGCAA AATCTCCTGC ACGTCTCCTG AGTTGTCTTG ATAGGCGATC    8880
```

```
TCGGCCATGA ACTGCTCGAT CTCTTCCTCC TGGAGATCTC CGCGTCCGGC TCGCTCCACG    8940

GTGGCGGCGA GGTCGTTGGA AATGCGGGCC ATGAGCTGCG AGAAGGCGTT GAGGCCTCCC    9000

TCGTTCCAGA CGCGGCTGTA GACCACGCCC CCTTCGGCAT CGCGGGCGCG CATGACCACC    9060

TGCGCGAGAT TGAGCTCCAC GTGCCGGGCG AAGACGGCGT AGTTTCGCAG GCGCTGAAAG    9120

AGGTAGTTGA GGGTGGTGGC GGTGTGTTCT GCCACGAAGA AGTACATAAC CCAGCGTCGC    9180

AACGTGGATT CGTTGATATC CCCCAAGGCC TCAAGGCGCT CCATGGCCTC GTAGAAGTCC    9240

ACGGCGAAGT TGAAAAACTG GGAGTTGCGC GCCGACACGG TTAACTCCTC CTCCAGAAGA    9300

CGGATGAGCT CGGCGACAGT GTCGCGCACC TCGCGCTCAA AGGCTACAGG GGCCTCTTCT    9360

TCTTCTTCAA TCTCCTCTTC CATAAGGGCC TCCCCTTCTT CTTCTTCTGG CGGCGGTGGG    9420

GGAGGGGGGA CACGGCGGCG ACGACGGCGC ACCGGGAGGC GGTCGACAAA GCGCTCGATC    9480

ATCTCCCCGC GGCGACGGCG CATGGTCTCG GTGACGGCGC GGCCGTTCTC GCGGGGGCGC    9540

AGTTGGAAGA CGCCGCCCGT CATGTCCCGG TTATGGGTTG GCGGGGGGCT GCCATGCGGC    9600

AGGGATACGG CGCTAACGAT GCATCTCAAC AATTGTTGTG TAGGTACTCC GCCGCCGAGG    9660

GACCTGAGCG AGTCCGCATC GACCGGATCG GAAAACCTCT CGAGAAAGGC GTCTAACCAG    9720

TCACAGTCGC AAGGTAGGCT GAGCACCGTG GCGGGCGGCA GCGGGCGGCG GTCGGGGTTG    9780

TTTCTGGCGG AGGTGCTGCT GATGATGTAA TTAAAGTAGG CGGTCTTGAG ACGGCGGATG    9840

GTCGACAGAA GCACCATGTC CTTGGGTCCG GCCTGCTGAA TGCGCAGGCG GTCGGCCATG    9900

CCCCAGGCTT CGTTTTGACA TCGGCGCAGG TCTTTGTAGT AGTCTTGCAT GAGCCTTTCT    9960

ACCGGCACTT CTTCTTCTCC TTCCTCTTGT CCTGCATCTC TTGCATCTAT CGCTGCGGCG   10020

GCGGCGGAGT TTGGCCGTAG GTGGCGCCCT CTTCCTCCCA TGCGTGTGAC CCCGAAGCCC   10080

CTCATCGGCT GAAGCAGGGC TAGGTCGGCG ACAACGCGCT CGGCTAATAT GGCCTGCTGC   10140

ACCTGCGTGA GGGTAGACTG GAAGTCATCC ATGTCCACAA AGCGGTGGTA TGCGCCCGTG   10200

TTGATGGTGT AAGTGCAGTT GGCCATAACG GACCAGTTAA CGGTCTGGTG ACCCGGCTGC   10260

GAGAGCTCGG TGTACCTGAG ACGCGAGTAA GCCCTCGAGT CAAATACGTA GTCGTTGCAA   10320

GTCCGCACCA GGTACTGGTA TCCCACCAAA AAGTGCGGCG GCGGCTGGCG GTAGAGGGGC   10380

CAGCGTAGGG TGGCCGGGGC TCCGGGGGCG AGATCTTCCA ACATAAGGCG ATGATATCCG   10440

TAGATGTACC TGGACATCCA GGTGATGCCG GCGGCGGTGG TGGAGGCGCG CGGAAAGTCG   10500

CGGACGCGGT TCCAGATGTT GCGCAGCGGC AAAAAGTGCT CCATGGTCGG GACGCTCTGG   10560

CCGGTCAGGC GCGCGCAATC GTTGACGCTC TAGACCGTGC AAAAGGAGAG CCTGTAAGCG   10620

GGCACTCTTC CGTGGTCTGG TGGATAAATT CGCAAGGGTA TCATGGCGGA CGACCGGGGT   10680

TCGAGCCCCG TATCCGGCCG TCCGCCGTGA TCCATGCGGT TACCGCCCGC GTGTCGAACC   10740

CAGGTGTGCG ACGTCAGACA ACGGGGGAGT GCTCCTTTTG GCTTCCTTCC AGGCGCGGCG   10800

GCTGCTGCGC TAGCTTTTTT GGCCACTGGC CGCGCGCAGC GTAAGCGGTT AGGCTGGAAA   10860

GCGAAAGCAT TAAGTGGCTC GCTCCCTGTA GCCGGAGGGT TATTTTCCAA GGGTTGAGTC   10920

GCGGGACCCC CGGTTCGAGT CTCGGACCGG CCGGACTGCG GCGAACGGGG GTTTGCCTCC   10980
```

```
CCGTCATGCA AGACCGGCCT TGCAAATTCC TCCGGAAACA GGGACGAGCC CCTTTTTTGC   11040
TTTTCCCAGA TGCATCCGGT GCTGCGGCAG ATGCGCCCCC CTCCTCAGCA GCGGCAAGAG   11100
CAAGAGCAGC GGCAGACATG CAGGGCACCC TCCCCTCCTC CTACCGCGTC AGGAGGGGCG   11160
ACATCCGCGG TTGACGCGGC AGCAGATGGT GATTACGAAC CCCCGCGGCG CCGGGCCCGG   11220
CACTACCTGG ACTTGGAGGA GGGCGAGGGC CTGGCGCGGC TAGGAGCGCC CTCTCCTGAG   11280
CGGTACCCAA GGGTGCAGCT GAAGCGTGAT ACGCGTGAGG CGTACGTGCC GCGGCAGAAC   11340
CTGTTTCGCG ACCGCGAGGG AGAGGAGCCC GAGGAGATGC GGGATCGAAA GTTCCACGCA   11400
GGGCGCGAGC TGCGGCATGG CCTGAATCGC GAGCGGTTGC TGCGCGAGGA GGACTTTGAG   11460
CCCGACGCGC GAACCGGGAT TAGTCCCGCG CGCGCACACG TGGCGGCCGC CGACCTGGTA   11520
ACCGCATACG AGCAGACGGT GAACCAGGAG ATTAACTTTC AAAAAAGCTT TAACAACCAC   11580
GTGCGTACGC TTGTGGCGCG CGAGGAGGTG GCTATAGGAC TGATGCATCT GTGGGACTTT   11640
GTAAGCGCGC TGGAGCAAAA CCCAAATAGC AAGCCGCTCA TGGCGCAGCT GTTCCTTATA   11700
GTGCAGCACA GCAGGGACAA CGAGGCATTC AGGGATGCGC TGCTAAACAT AGTAGAGCCC   11760
GAGGGCCGCT GGCTGCTCGA TTTGATAAAC ATCCTGCAGA GCATAGTGGT GCAGGAGCGC   11820
AGCTTGAGCC TGGCTGACAA GGTGGCCGCC ATCAACTATT CCATGCTTAG CCTGGGCAAG   11880
TTTTACGCCC GCAAGATATA CCATACCCCT TACGTTCCCA TAGACAAGGA GGTAAAGATC   11940
GAGGGGTTCT ACATGCGCAT GGCGCTGAAG GTGCTTACCT TGAGCGACGA CCTGGGCGTT   12000
TATCGCAACG AGCGCATCCA CAAGGCCGTG AGCGTGAGCC GGCGGCGCGA GCTCAGCGAC   12060
CGCGAGCTGA TGCACAGCCT GCAAAGGGCC CTGGCTGGCA CGGGCAGCGG CGATAGAGAG   12120
GCCGAGTCCT ACTTTGACGC GGGCGCTGAC CTGCGCTGGG CCCCAAGCCG ACGCGCCCTG   12180
GAGGCAGCTG GGGCCGGACC TGGGCTGGCG GTGGCACCCG CGCGCGCTGG CAACGTCGGC   12240
GGCGTGGAGG AATATGACGA GGACGATGAG TACGAGCCAG AGGACGGCGA GTACTAAGCG   12300
GTGATGTTTC TGATCAGATG ATGCAAGACG CAACGGACCC GGCGGTGCGG GCGGCGCTGC   12360
AGAGCCAGCC GTCCGGCCTT AACTCCACGG ACGACTGGCG CCAGGTCATG GACCGCATCA   12420
TGTCGCTGAC TGCGCGCAAT CCTGACGCGT TCCGGCAGCA GCCGCAGGCC AACCGGCTCT   12480
CCGCAATTCT GGAAGCGGTG GTCCCGGCGC GCGCAAACCC CACGCACGAG AAGGTGCTGG   12540
CGATCGTAAA CGCGCTGGCC GAAAACAGGG CCATCCGGCC CGACGAGGCC GGCCTGGTCT   12600
ACGACGCGCT GCTTCAGCGC GTGGCTCGTT ACAACAGCGG CAACGTGCAG ACCAACCTGG   12660
ACCGGCTGGT GGGGGATGTG CGCGAGGCCG TGGCGCAGCG TGAGCGCGCG CAGCAGCAGG   12720
GCAACCTGGG CTCCATGGTT GCACTAAACG CCTTCCTGAG TACACAGCCC GCCAACGTGC   12780
CGCGGGGACA GGAGGACTAC ACCAACTTTG TGAGCGCACT GCGGCTAATG GTGACTGAGA   12840
CACCGCAAAG TGAGGTGTAC CAGTCTGGGC AGACTATTT TTTCCAGACC AGTAGACAAG   12900
GCCTGCAGAC CGTAAACCTG AGCCAGGCTT TCAAAAACTT GCAGGGGCTG TGGGGGGTGC   12960
GGGCTCCCAC AGGCGACCGC GCGACCGTGT CTAGCTTGCT GACGCCCAAC TCGCGCCTGT   13020
TGCTGCTGCT AATAGCGCCC TTCACGGACA GTGGCAGCGT GTCCCGGGAC ACATACCTAG   13080
```

```
GTCACTTGCT GACACTGTAC CGCGAGGCCA TAGGTCAGGC GCATGTGGAC GAGCATACTT   13140

TCCAGGAGAT TACAAGTGTC AGCCGCGCGC TGGGGCAGGA GGACACGGGC AGCCTGGAGG   13200

CAACCCTAAA CTACCTGCTG ACCAACCGGC GGCAGAAGAT CCCCTCGTTG CACAGTTTAA   13260

ACAGCGAGGA GGAGCGCATT TTGCGCTACG TGCAGCAGAG CGTGAGCCTT AACCTGATGC   13320

GCGACGGGGT AACGCCCAGC GTGGCGCTGG ACATGACCGC GCGCAACATG GAACCGGGCA   13380

TGTATGCCTC AAACCGGCCG TTTATCAACC GCCTAATGGA CTACTTGCAT CGCGCGGCCG   13440

CCGTGAACCC CGAGTATTTC ACCAATGCCA TCTTGAACCC GCACTGGCTA CCGCCCCCTG   13500

GTTTCTACAC CGGGGGATTC GAGGTGCCCG AGGGTAACGA TGGATTCCTC TGGGACGACA   13560

TAGACGACAG CGTGTTTTCC CCGCAACCGC AGACCCTGCT AGAGTTGCAA CAGCGCGAGC   13620

AGGCAGAGGC GGCGCTGCGA AAGGAAAGCT TCCGCAGGCC AAGCAGCTTG TCCGATCTAG   13680

GCGCTGCGGC CCCGCGGTCA GATGCTAGTA GCCCATTTCC AAGCTTGATA GGGTCTCTTA   13740

CCAGCACTCG CACCACCCGC CCGCGCCTGC TGGGCGAGGA GGAGTACCTA AACAACTCGC   13800

TGCTGCAGCC GCAGCGCGAA AAAAACCTGC CTCCGGCATT TCCCAACAAC GGGATAGAGA   13860

GCCTAGTGGA CAAGATGAGT AGATGGAAGA CGTACGCGCA GGAGCACAGG GACGTGCCAG   13920

GCCCGCGCCC GCCCACCCGT CGTCAAAGGC ACGACCGTCA GCGGGGTCTG GTGTGGGAGG   13980

ACGATGACTC GGCAGACGAC AGCAGCGTCC TGGATTTGGG AGGGAGTGGC AACCCGTTTG   14040

CGCACCTTCG CCCCAGGCTG GGGAGAATGT TTTAAAAAAA AAAAAGCATG ATGCAAAATA   14100

AAAAACTCAC CAAGGCCATG GCACCGAGCG TTGGTTTTCT TGTATTCCCC TTAGTATGCG   14160

GCGCGCGGCG ATGTATGAGG AAGGTCCTCC TCCCTCCTAC GAGAGTGTGG TGAGCGCGGC   14220

GCCAGTGGCG GCGGCGCTGG GTTCTCCCTT CGATGCTCCC CTGGACCCGC CGTTTGTGCC   14280

TCCGCGGTAC CTGCGGCCTA CCGGGGGGAG AAACAGCATC CGTTACTCTG AGTTGGCACC   14340

CCTATTCGAC ACCACCCGTG TGTACCTGGT GGACAACAAG TCAACGGATG TGGCATCCCT   14400

GAACTACCAG AACGACCACA GCAACTTTCT GACCACGGTC ATTCAAAACA ATGACTACAG   14460

CCCGGGGGAG GCAAGCACAC AGACCATCAA TCTTGACGAC CGGTCGCACT GGGGCGGCGA   14520

CCTGAAAACC ATCCTGCATA CCAACATGCC AAATGTGAAC GAGTTCATGT TTACCAATAA   14580

GTTTAAGGCG CGGGTGATGG TGTCGCGCTT GCCTACTAAG GACAATCAGG TGGAGCTGAA   14640

ATACGAGTGG GTGGAGTTCA CGCTGCCCGA GGGCAACTAC TCCGAGACCA TGACCATAGA   14700

CCTTATGAAC AACGCGATCG TGGAGCACTA CTTGAAAGTG GGCAGACAGA ACGGGGTTCT   14760

GGAAAGCGAC ATCGGGGTAA AGTTTGACAC CCGCAACTTC AGACTGGGGT TTGACCCCGT   14820

CACTGGTCTT GTCATGCCTG GGGTATATAC AAACGAAGCC TTCCATCCAG ACATCATTTT   14880

GCTGCCAGGA TGCGGGGTGG ACTTCACCCA CAGCCGCCTG AGCAACTTGT TGGGCATCCG   14940

CAAGCGGCAA CCCTTCCAGG AGGGCTTTAG GATCACCTAC GATGATCTGG AGGGTGGTAA   15000

CATTCCCGCA CTGTTGGATG TGGACGCCTA CCAGGCGAGC TTGAAAGATG ACACCGAACA   15060

GGGCGGGGGT GGCGCAGGCG GCAGCAACAG CAGTGGCAGC GGCGCGGAAG AGAACTCCAA   15120

CGCGGCAGCC GCGGCAATGC AGCCGGTGGA GGACATGAAC GATCATGCCA TTCGCGGCGA   15180
```

```
CACCTTTGCC ACACGG    G AGGAGAAGCG CGCTGAGGCC GAAGCAGCGG CCGAAGCTGC    15240
CGCCCCCGCT GCGCAACCCG AGGTCGAGAA GCCTCAGAAG AAACCGGTGA TCAAACCCCT    15300
GACAGAGGAC AGCAAGAAAC GCAGTTACAA CCTAATAAGC AATGACAGCA CCTTCACCCA    15360
GTACCGCAGC TGGTACCTTG CATACAACTA CGGCGACCCT CAGACCGGAA TCCGCTCATG    15420
GACCCTGCTT TGCACTCCTG ACGTAACCTG CGGCTCGGAG CAGGTCTACT GGTCGTTGCC    15480
AGACATGATG CAAGACCCCG TGACCTTCCG CTCCACGCGC CAGATCAGCA ACTTTCCGGT    15540
GGTGGGCGCC GAGCTGTTGC CCGTGCACTC CAAGAGCTTC TACAACGACC AGGCCGTCTA    15600
CTCCCAACTC ATCCGCCAGT TTACCTCTCT GACCCACGTG TTCAATCGCT TTCCCGAGAA    15660
CCAGATTTTG GCGCGCCCGC CAGCCCCCAC CATCACCACC GTCAGTGAAA ACGTTCCTGC    15720
TCTCACAGAT CACGGGACGC TACCGCTGCG CAACAGCATC GGAGGAGTCC AGCGAGTGAC    15780
CATTACTGAC GCCAGACGCC GCACCTGCCC CTACGTTTAC AAGGCCCTGG GCATAGTCTC    15840
GCCGCGCGTC CTATCGAGCC GCACTTTTTG AGCAAGCATG TCCATCCTTA TATCGCCCAG    15900
CAATAACACA GGCTGGGGCC TGCGCTTCCC AAGCAAGATG TTTGGCGGGG CCAAGAAGCG    15960
CTCCGACCAA CACCCAGTGC GCGTGCGCGG GCACTACCGC GCGCCCTGGG GCGCGCACAA    16020
ACGCGGCCGC ACTGGGCGCA CCACCGTCGA TGACGCCATC GACGCGGTGG TGGAGGAGGC    16080
GCGCAACTAC ACGCCCACGC CGCCACCAGT GTCCACAGTG GACGCGGCCA TTCAGACCGT    16140
GGTGCGCGGA GCCCGGCGCT ATGCTAAAAT GAAGAGACGG CGGAGGCGCG TAGCACGTCG    16200
CCACCGCCGC CGACCCGGCA CTGCCGCCCA ACGCGCGGCG GCGGCCCTGC TTAACCGCGC    16260
ACGTCGCACC GGCCGACGGG CGGCCATGCG GGCCGCTCGA AGGCTGGCCG CGGGTATTGT    16320
CACTGTGCCC CCCAGGTCCA GGCGACGAGC GGCCGCCGCA GCAGCCGCGG CCATTAGTGC    16380
TATGACTCAG GGTCGCAGGG GCAACGTGTA TTGGGTGCGC GACTCGGTTA GCGGCCTGCG    16440
CGTGCCCGTG CGCACCCGCC CCCCGCGCAA CTAGATTGCA AGAAAAAACT ACTTAGACTC    16500
GTACTGTTGT ATGTATCCAG CGGCGGCGGC GCGCAACGAA GCTATGTCCA AGCGCAAAAT    16560
CAAAGAAGAG ATGCTCCAGG TCATCGCGCC GGAGATCTAT GGCCCCCCGA AGAAGGAAGA    16620
GCAGGATTAC AAGCCCCGAA AGCTAAAGCG GGTCAAAAAG AAAAAGAAAG ATGATGATGA    16680
TGAACTTGAC GACGAGGTGG AACTGCTGCA CGCTACCGCG CCCAGGCGAC GGGTACAGTG    16740
GAAAGGTCGA CGCGTAAAAC GTGTTTTGCG ACCCGGCACC ACCGTAGTCT TTACGCCCGG    16800
TGAGCGCTCC ACCCGCACCT ACAAGCGCGT GTATGATGAG GTGTACGGCG ACGAGGACCT    16860
GCTTGAGCAG GCCAACGAGC GCCTCGGGGA GTTTGCCTAC GGAAAGCGGC ATAAGGACAT    16920
GCTGGCGTTG CCGCTGGACG AGGGCAACCC AACACCTAGC CTAAAGCCCG TAACACTGCA    16980
GCAGGTGCTG CCCGCGCTTG CACCGTCCGA AGAAAAGCGC GGCCTAAAGC GCGAGTCTGG    17040
TGACTTGGCA CCCACCGTGC AGCTGATGGT ACCCAAGCGC CAGCGACTGG AAGATGTCTT    17100
GGAAAAAATG ACCGTGGAAC CTGGGCTGGA GCCCGAGGTC CGCGTGCGGC CAATCAAGCA    17160
GGTGGCGCCG GGACTGGGCG TGCAGACCGT GGACGTTCAG ATACCCACTA CCAGTAGCAC    17220
CAGTATTGCC ACCGCCACAG AGGGCATGGA GACACAAACG TCCCCGGTTG CCTCAGCGGT    17280
```

```
GGCGGATGCC GCGGTGCGCG CGGTCGCTGC GGCCGCGTCC AAGACCTCTA CGGAGGTGCA    17340
AACGGACCCG TGGATGTTTC GCGTTTCAGC CCCCCGGCGC CCGCGCGGTT CGAGGAAGTA    17400
CGGCGCCGCC AGCGCGCTAC TGCCCGAATA TGCCCTACAT CCTTCCATTG CGCCTACCCC    17460
CGGCTATCGT GGCTACACCT ACCGCCCCAG AAGACGAGCA ACTACCCGAC GCCGAACCAC    17520
CACTGGAACC CGCCGCCGCC GTCGCCGTCG CCAGCCCGTG CTGGCCCCGA TTTCCGTGCG    17580
CAGGGTGGCT CGCGAAGGAG GCAGGACCCT GGTGCTGCCA ACAGCGCGCT ACCACCCCAG    17640
CATCGTTTAA AAGCCGGTCT TTGTGGTTCT TGCAGATATG GCCCTCACCT GCCGCCTCCG    17700
TTTCCCGGTG CCGGGATTCC GAGGAAGAAT GCACCGTAGG AGGGGCATGG CCGGCCACGG    17760
CCTGACGGGC GGCATGCGTC GTGCGCACCA CCGGCGGCGG CGCGCGTCGC ACCGTCGCAT    17820
GCGCGGCGGT ATCCTGCCCC TCCTTATTCC ACTGATCGCC GCGGCGATTG GCGCCGTGCC    17880
CGGAATTGCA TCCGTGGCCT TGCAGGCGCA GAGACACTGA TTAAAAACAA GTTGCATGTG    17940
GAAAAATCAA AATAAAAAGT CTGGACTCTC ACGCTCGCTT GGTCCTGTAA CTATTTTGTA    18000
GAATGGAAGA CATCAACTTT GCGTCTCTGG CCCCGCGACA CGGCTCGCGC CCGTTCATGG    18060
GAAACTGGCA AGATATCGGC ACCAGCAATA TGAGCGGTGG CGCCTTCAGC TGGGGCTCGC    18120
TGTGGAGCGG CATTAAAAAT TTCGGTTCCA CCGTTAAGAA CTATGGCAGC AAGGCCTGGA    18180
ACAGCAGCAC AGGCCAGATG CTGAGGGATA AGTTGAAAGA GCAAAATTTC CAACAAAGG    18240
TGGTAGATGG CCTGGCCTCT GGCATTAGCG GGGTGGTGGA CCTGGCCAAC CAGGCAGTGC    18300
AAAATAAGAT TAACAGTAAG CTTGATCCCC GCCCTCCCGT AGAGGAGCCT CCACCGGCCG    18360
TGGAGACAGT GTCTCCAGAG GGGCGTGGCG AAAAGCGTCC GCGCCCCGAC AGGGAAGAAA    18420
CTCTGGTGAC GCAAATAGAC GAGCCTCCCT CGTACGAGGA GGCACTAAAG CAAGGCCTGC    18480
CCACCACCCG TCCCATCGCG CCCATGGCTA CCGGAGTGCT GGGCCAGCAC ACACCCGTAA    18540
CGCTGGACCT GCCTCCCCCC GCCGACACCC AGCAGAAACC TGTGCTGCCA GGCCCGACCG    18600
CCGTTGTTGT AACCCGTCCT AGCCGCGCGT CCCTGCGCCG CGCCGCCAGC GGTCCGCGAT    18660
CGTTGCGGCC CGTAGCCAGT GGCAACTGGC AAAGCACACT GAACAGCATC GTGGGTCTGG    18720
GGGTGCAATC CCTGAAGCGC CGACGATGCT TCTGAATAGC TAACGTGTCG TATGTGTGTC    18780
ATGTATGCGT CCATGTCGCC GCCAGAGGAG CTGCTGAGCC GCCGCGCGCC CGCTTTCCAA    18840
GATGGCTACC CCTTCGATGA TGCCGCAGTG GTCTTACATG CACATCTCGG GCCAGGACGC    18900
CTCGGAGTAC CTGAGCCCCG GGCTGGTGCA GTTTGCCCGC GCCACCGAGA CGTACTTCAG    18960
CCTGAATAAC AAGTTTAGAA ACCCCACGGT GGCGCCTACG CACGACGTGA CCACAGACCG    19020
GTCCAGCGT TTGACGCTGC GGTTCATCCC TGTGGACCGT GAGGATACTG CGTACTCGTA    19080
CAAGGCGCGG TTCACCCTAG CTGTGGGTGA TAACCGTGTG CTGGACATGG CTTCCACGTA    19140
CTTTGACATC CGCGGCGTGC TGGACAGGGG CCCTACTTTT AAGCCCTACT CTGGCACTGC    19200
CTACAACGCC CTGGCTCCCA AGGGTGCCCC AAATCCTTGC GAATGGGATG AAGCTGCTAC    19260
TGCTCTTGAA ATAAACCTAG AAGAAGAGGA CGATGACAAC GAAGACGAAG TAGACGAGCA    19320
AGCTGAGCAG CAAAAAACTC ACGTATTTGG GCAGGCGCCT TATTCTGGTA TAAATATTAC    19380
```

```
AAAGGAGGGT ATTCAAATAG GTGTCGAAGG TCAAACACCT AAATATGCCG ATAAAACATT   19440

TCAACCTGAA CCTCAAATAG GAGAATCTCA GTGGTACGAA ACTGAAATTA ATCATGCAGC   19500

TGGGAGAGTC CTTAAAAAGA CTACCCCAAT GAAACCATGT TACGGTTCAT ATGCAAAACC   19560

CACAAATGAA AATGGAGGGC AAGGCATTCT TGTAAAGCAA CAAAATGGAA AGCTAGAAAG   19620

TCAAGTGGAA ATGCAATTTT TCTCAACTAC TGAGGCGACC GCAGGCAATG GTGATAACTT   19680

GACTCCTAAA GTGGTATTGT ACAGTGAAGA TGTAGATATA GAAACCCCAG ACACTCATAT   19740

TTCTTACATG CCCACTATTA AGGAAGGTAA CTCACGAGAA CTAATGGGCC AACAATCTAT   19800

GCCCAACAGG CCTAATTACA TTGCTTTTAG GGACAATTTT ATTGGTCTAA TGTATTACAA   19860

CAGCACGGGT AATATGGGTG TTCTGGCGGG CCAAGCATCG CAGTTGAATG CTGTTGTAGA   19920

TTTGCAAGAC AGAAACACAG AGCTTTCATA CCAGCTTTTG CTTGATTCCA TTGGTGATAG   19980

AACCAGGTAC TTTTCTATGT GGAATCAGGC TGTTGACAGC TATGATCCAG ATGTTAGAAT   20040

TATTGAAAAT CATGGAACTG AAGATGAACT TCCAAATTAC TGCTTTCCAC TGGGAGGTGT   20100

GATTAATACA GAGACTCTTA CCAAGGTAAA ACCTAAAACA GGTCAGGAAA ATGGATGGGA   20160

AAAAGATGCT ACAGAATTTT CAGATAAAAA TGAAATAAGA GTTGGAAATA ATTTTGCCAT   20220

GGAAATCAAT CTAAATGCCA ACCTGTGGAG AAATTTCCTG TACTCCAACA TAGCGCTGTA   20280

TTTGCCCGAC AAGCTAAAGT ACAGTCCTTC CAACGTAAAA ATTTCTGATA ACCCAAACAC   20340

CTACGACTAC ATGAACAAGC GAGTGGTGGC TCCCGGGTTA GTGGACTGCT ACATTAACCT   20400

TGGAGCACGC TGGTCCCTTG ACTATATGGA CAACGTCAAC CCATTTAACC ACCACCGCAA   20460

TGCTGGCCTG CGCTACCGCT CAATGTTGCT GGGCAATGGT CGCTATGTGC CCTTCCACAT   20520

CCAGGTGCCT CAGAAGTTCT TTGCCATTAA AAACCTCCTT CTCCTGCCGG GCTCATACAC   20580

CTACGAGTGG AACTTCAGGA AGGATGTTAA CATGGTTCTG CAGAGCTCCC TAGGAAATGA   20640

CCTAAGGGTT GACGGAGCCA GCATTAAGTT TGATAGCATT TGCCTTTACG CCACCTTCTT   20700

CCCCATGGCC CACAACACCG CCTCCACGCT TGAGGCCATG CTTAGAAACG ACACCAACGA   20760

CCAGTCCTTT AACGACTATC TCTCCGCCGC CAACATGCTC TACCCTATAC CCGCCAACGC   20820

TACCAACGTG CCCATATCCA TCCCCTCCCG CAACTGGGCG GCTTTCCGCG GCTGGGCCTT   20880

CACGCGCCTT AAGACTAAGG AAACCCCATC ACTGGGCTCG GGCTACGACC CTTATTACAC   20940

CTACTCTGGC TCTATACCCT ACCTAGATGG AACCTTTTAC CTCAACCACA CCTTTAAGAA   21000

GGTGGCCATT ACCTTTGACT CTTCTGTCAG CTGGCCTGGC AATGACCGCC TGCTTACCCC   21060

CAACGAGTTT GAAATTAAGC GCTCAGTTGA CGGGGAGGGT TACAACGTTG CCCAGTGTAA   21120

CATGACCAAA GACTGGTTCC TGGTACAAAT GCTAGCTAAC TACAACATTG CTACCAGGG   21180

CTTCTATATC CCAGAGAGCT ACAAGGACCG CATGTACTCC TTCTTTAGAA ACTTCCAGCC   21240

CATGAGCCGT CAGGTGGTGG ATGATACTAA ATACAAGGAC TACCAACAGG TGGGCATCCT   21300

ACACCAACAC AACAACTCTG GATTTGTTGG CTACCTTGCC CCCACCATGC GCGAAGGACA   21360

GGCCTACCCT GCTAACTTCC CCTATCCGCT TATAGGCAAG ACCGCAGTTG ACAGCATTAC   21420

CCAGAAAAAG TTTCTTTGCG ATCGCACCCT TTGGCGCATC CCATTCTCCA GTAACTTTAT   21480
```

```
GTCCATGGGC GCACTCACAG ACCTGGGCCA AAACCTTCTC TACGCCAACT CCGCCCACGC     21540

GCTAGACATG ACTTTTGAGG TGGATCCCAT GGACGAGCCC ACCCTTCTTT ATGTTTTGTT     21600

TGAAGTCTTT GACGTGGTCC GTGTGCACCG GCCGCACCGC GGCGTCATCG AAACCGTGTA     21660

CCTGCGCACG CCCTTCTCGG CCGGCAACGC CACAACATAA AGAAGCAAGC AACATCAACA     21720

ACAGCTGCCG CCATGGGCTC CAGTGAGCAG GAACTGAAAG CCATTGTCAA AGATCTTGGT     21780

TGTGGGCCAT ATTTTTTGGG CACCTATGAC AAGCGCTTTC CAGGCTTTGT TTCTCCACAC     21840

AAGCTCGCCT GCGCCATAGT CAATACGGCC GGTCGCGAGA CTGGGGGCGT ACACTGGATG     21900

GCCTTTGCCT GGAACCCGCA CTCAAAAACA TGCTACCTCT TTGAGCCCTT TGGCTTTTCT     21960

GACCAGCGAC TCAAGCAGGT TTACCAGTTT GAGTACGAGT CACTCCTGCG CCGTAGCGCC     22020

ATTGCTTCTT CCCCCGACCG CTGTATAACG CTGGAAAAGT CCACCCAAAG CGTACAGGGG     22080

CCCAACTCGG CCGCCTGTGG ACTATTCTGC TGCATGTTTC TCCACGCCTT TGCCAACTGG     22140

CCCCAAACTC CCATGGATCA CAACCCCACC ATGAACCTTA TTACCGGGGT ACCCAACTCC     22200

ATGCTCAACA GTCCCCAGGT ACAGCCCACC CTGCGTCGCA ACCAGGAACA GCTCTACAGC     22260

TTCCTGGAGC GCCACTCGCC CTACTTCCGC AGCCACAGTG CGCAGATTAG GAGCGCCACT     22320

TCTTTTTGTC ACTTGAAAAA CATGTAAAAA TAATGTACTA GAGACACTTT CAATAAAGGC     22380

AAATGCTTTT ATTTGTACAC TCTCGGGTGA TTATTTACCC CCACCCTTGC CGTCTGCGCC     22440

GTTTAAAAAT CAAAGGGGTT CTGCCGCGCA TCGCTATGCG CCACTGGCAG GGACACGTTG     22500

CGATACTGGT GTTTAGTGCT CCACTTAAAC TCAGGCACAA CCATCCGCGG CAGCTCGGTG     22560

AAGTTTTCAC TCCACAGGCT GCGCACCATC ACCAACGCGT TTAGCAGGTC GGGCGCCGAT     22620

ATCTTGAAGT CGCAGTTGGG GCCTCCGCCC TGCGCGCGCG AGTTGCGATA CACAGGGTTG     22680

CAGCACTGGA ACACTATCAG CGCCGGGTGG TGCACGCTGG CCAGCACGCT CTTGTCGGAG     22740

ATCAGATCCG CGTCCAGGTC CTCCGCGTTG CTCAGGGCGA ACGGAGTCAA CTTTGGTAGC     22800

TGCCTTCCCA AAAAGGGCGC GTGCCCAGGC TTTGAGTTGC ACTCGCACCG TAGTGGCATC     22860

AAAAGGTGAC CGTGCCCGGT CTGGGCGTTA GGATACAGCG CCTGCATAAA AGCCTTGATC     22920

TGCTTAAAAG CCACCTGAGC CTTTGCGCCT TCAGAGAAGA ACATGCCGCA AGACTTGCCG     22980

GAAAACTGAT TGGCCGGACA GGCCGCGTCG TGCACGCAGC ACCTTGCGTC GGTGTTGGAG     23040

ATCTGCACCA CATTTCGGCC CCACCGGTTC TTCACGATCT TGGCCTTGCT AGACTGCTCC     23100

TTCAGCGCGC GCTGCCCGTT TTCGCTCGTC ACATCCATTT CAATCACGTG CTCCTTATTT     23160

ATCATAATGC TTCCGTGTAG ACACTTAAGC TCGCCTTCGA TCTCAGCGCA GCGGTGCAGC     23220

CACAACGCGC AGCCCGTGGG CTCGTGATGC TTGTAGGTCA CCTCTGCAAA CGACTGCAGG     23280

TACGCCTGCA GGAATCGCCC CATCATCGTC ACAAAGGTCT TGTTGCTGGT GAAGGTCAGC     23340

TGCAACCCGC GGTGCTCCTC GTTCAGCCAG GTCTTGCATA CGGCCGCCAG AGCTTCCACT     23400

TGGTCAGGCA GTAGTTTGAA GTTCGCCTTT AGATCGTTAT CCACGTGGTA CTTGTCCATC     23460

AGCGCGCGCG CAGCCTCCAT GCCCTTCTCC CACGCAGACA CGATCGGCAC ACTCAGCGGG     23520

TTCATCACCG TAATTTCACT TTCCGCTTCG CTGGGCTCTT CCTCTTCCTC TTGCGTCCGC     23580
```

```
ATACCACGCG CCACTGGGTC GTCTTCATTC AGCCGCCGCA CTGTGCGCTT ACCTCCTTTG    23640
CCATGCTTGA TTAGCACCGG TGGGTTGCTG AAACCCACCA TTTGTAGCGC CACATCTTCT    23700
CTTTCTTCCT CGCTGTCCAC GATTACCTCT GGTGATGGCG GGCGCTCGGG CTTGGGAGAA    23760
GGGCGCTTCT TTTTCTTCTT GGGCGCAATG GCCAAATCCG CCGCCGAGGT CGATGGCCGC    23820
GGGCTGGGTG TGCGCGGCAC CAGCGCGTCT TGTGATGAGT CTTCCTCGTC CTCGGACTCG    23880
ATACGCCGCC TCATCCGCTT TTTTGGGGGC GCCCGGGGAG GCGGCGGCGA CGGGGACGGG    23940
GACGACACGT CCTCCATGGT TGGGGGACGT CGCGCCGCAC CGCGTCCGCG CTCGGGGGTG    24000
GTTTCGCGCT GCTCCTCTTC CCGACTGGCC ATTTCCTTCT CCTATAGGCA GAAAAAGATC    24060
ATGGAGTCAG TCGAGAAGAA GGACAGCCTA ACCGCCCCCT CTGAGTTCGC CACCACCGCC    24120
TCCACCGATG CCGCCAACGC GCCTACCACC TTCCCCGTCG AGGCACCCCC GCTTGAGGAG    24180
GAGGAAGTGA TTATCGAGCA GGACCCAGGT TTTGTAAGCG AAGACGACGA GGACCGCTCA    24240
GTACCAACAG AGGATAAAAA GCAAGACCAG GACAACGCAG AGGCAAACGA GGAACAAGTC    24300
GGGCGGGGGG ACGAAAGGCA TGGCGACTAC CTAGATGTGG GAGACGACGT GCTGTTGAAG    24360
CATCTGCAGC GCCAGTGCGC CATTATCTGC GACGCGTTGC AAGAGCGCAG CGATGTGCCC    24420
CTCGCCATAG CGGATGTCAG CCTTGCCTAC GAACGCCACC TATTCTCACC GCGCGTACCC    24480
CCCAAACGCC AAGAAACGG CACATGCGAG CCCAACCCGC GCCTCAACTT CTACCCCGTA    24540
TTTGCCGTGC CAGAGGTGCT TGCCACCTAT CACATCTTTT CCAAAACTG CAAGATACCC    24600
CTATCCTGCC GTGCCAACCG CAGCCGAGCG GACAAGCAGC TGGCCTTGCG GCAGGGCGCT    24660
GTCATACCTG ATATCGCCTC GCTCAACGAA GTGCCAAAAA TCTTTGAGGG TCTTGGACGC    24720
GACGAGAAGC GCGCGGCAAA CGCTCTGCAA CAGGAAAACA GCGAAAATGA AAGTCACTCT    24780
GGAGTGTTGG TGGAACTCGA GGGTGACAAC GCGCGCCTAG CCGTACTAAA ACGCAGCATC    24840
GAGGTCACCC ACTTTGCCTA CCCGGCACTT AACCTACCCC CCAAGGTCAT GAGCACAGTC    24900
ATGAGTGAGC TGATCGTGCG CCGTGCGCAG CCCCTGGAGA GGGATGCAAA TTTGCAAGAA    24960
CAAACAGAGG AGGGCCTACC CGCAGTTGGC GACGAGCAGC TAGCGCGCTG GCTTCAAACG    25020
CGCGAGCCTG CCGACTTGGA GGAGCGACGC AAACTAATGA TGGCCGCAGT GCTCGTTACC    25080
GTGGAGCTTG AGTGCATGCA GCGGTTCTTT GCTGACCCGG AGATGCAGCG CAAGCTAGAG    25140
GAAACATTGC ACTACACCTT TCGACAGGGC TACGTACGCC AGGCCTGCAA GATCTCCAAC    25200
GTGGAGCTCT GCAACCTGGT CTCCTACCTT GGAATTTTGC ACGAAAACCG CCTTGGGCAA    25260
AACGTGCTTC ATTCCACGCT CAAGGGCGAG GCGCGCCGCG ACTACGTCCG CGACTGCGTT    25320
TACTTATTTC TATGCTACAC CTGGCAGACG GCCATGGGCG TTTGGCAGCA GTGCTTGGAG    25380
GAGTGCAACC TCAAGGAGCT GCAGAAACTG CTAAAGCAAA ACTTGAAGGA CCTATGGACG    25440
GCCTTCAACG AGCGCTCCGT GGCCGCGCAC CTGGCGGACA TCATTTTCCC CGAACGCCTG    25500
CTTAAAACCC TGCAACAGGG TCTGCCAGAC TTCACCAGTC AAAGCATGTT GCAGAACTTT    25560
AGGAACTTTA TCCTAGAGCG CTCAGGAATC TTGCCCGCCA CCTGCTGTGC ACTTCCTAGC    25620
GACTTTGTGC CCATTAAGTA CCGCGAATGC CCTCCGCCGC TTTGGGGCCA CTGCTACCTT    25680
```

```
CTGCAGCTAG CCAACTACCT TGCCTACCAC TCTGACATAA TGGAAGACGT GAGCGGTGAC   25740

GGTCTACTGG AGTGTCACTG TCGCTGCAAC CTATGCACCC CGCACCGCTC CCTGGTTTGC   25800

AATTCGCAGC TGCTTAACGA AAGTCAAATT ATCGGTACCT TTGAGCTGCA GGGTCCCTCG   25860

CCTGACGAAA AGTCCGCGGC TCCGGGGTTG AAACTCACTC CGGGGCTGTG GACGTCGGCT   25920

TACCTTCGCA AATTTGTACC TGAGGACTAC CACGCCCACG AGATTAGGTT CTACGAAGAC   25980

CAATCCCGCC CGCCAAATGC GGAGCTTACC GCCTGCGTCA TTACCCAGGG CCACATTCTT   26040

GGCCAATTGC AAGCCATCAA CAAAGCCCGC CAAGAGTTTC TGCTACGAAA GGGACGGGGG   26100

GTTTACTTGG ACCCCCAGTC CGGCGAGGAG CTCAACCCAA TCCCCCCGCC GCCGCAGCCC   26160

TATCAGCAGC AGCCGCGGGC CCTTGCTTCC CAGGATGGCA CCCAAAAAGA AGCTGCAGCT   26220

GCCGCCGCCA CCCACGGACG AGGAGGAATA CTGGGACAGT CAGGCAGAGG AGGTTTTGGA   26280

CGAGGAGGAG GAGGACATGA TGGAAGACTG GGAGAGCCTA GACGAGGAAG CTTCCGAGGT   26340

CGAAGAGGTG TCAGACGAAA CACCGTCACC CTCGGTCGCA TTCCCCTCGC CGGCGCCCCA   26400

GAAATCGGCA ACCGGTTCCA GCATGGCTAC AACCTCCGCT CCTCAGGCGC CGCCGGCACT   26460

GCCCGTTCGC CGACCCAACC GTAGATGGGA CACCACTGGA ACCAGGGCCG GTAAGTCCAA   26520

GCAGCCGCCG CCGTTAGCCC AAGAGCAACA ACAGCGCCAA GGCTACCGCT CATGGCGCGG   26580

GCACAAGAAC GCCATAGTTG CTTGCTTGCA AGACTGTGGG GGCAACATCT CCTTCGCCCG   26640

CCGCTTTCTT CTCTACCATC ACGGCGTGGC CTTCCCCCGT AACATCCTGC ATTACTACCG   26700

TCATCTCTAC AGCCCATACT GCACCGGCGG CAGCGGCAGC GGCAGCAACA GCAGCGGCCA   26760

CACAGAAGCA AAGGCGACCG GATAGCAAGA CTCTGACAAA GCCCAAGAAA TCCACAGCGG   26820

CGGCAGCAGC AGGAGGAGGA GCGCTGCGTC TGGCGCCCAA CGAACCCGTA TCGACCCGCG   26880

AGCTTAGAAA CAGGATTTTT CCCACTCTGT ATGCTATATT TCAACAGAGC AGGGGCCAAG   26940

AACAAGAGCT GAAAATAAAA AACAGGTCTC TGCGATCCCT CACCCGCAGC TGCCTGTATC   27000

ACAAAAGCGA AGATCAGCTT CGGCGCACGC TGGAAGACGC GGAGGCTCTC TTCAGTAAAT   27060

ACTGCGCGCT GACTCTTAAG GACTAGTTTC GCGCCCTTTC TCAAATTTAA GCGCGAAAAC   27120

TACGTCATCT CCAGCGGCCA CACCCGGCGC CAGCACCTGT CGTCAGCGCC ATTATGAGCA   27180

AGGAAATTCC CACGCCCTAC ATGTGGAGTT ACCAGCCACA AATGGGACTT GCGGCTGGAG   27240

CTGCCCAAGA CTACTCAACC CGAATAAACT ACATGAGCGC GGGACCCCAC ATGATATCCC   27300

GGGTCAACGG AATCCGCGCC CACCGAAACC GAATTCTCTT GGAACAGGCG GCTATTACCA   27360

CCACACCTCG TAATAACCTT AATCCCCGTA GTTGGCCCGC TGCCCTGGTG TACCAGGAAA   27420

GTCCCGCTCC CACCACTGTG GTACTTCCCA GAGACGCCCA GGCCGAAGTT CAGATGACTA   27480

ACTCAGGGGC GCAGCTTGCG GGCGGCTTTC GTCACAGGGT GCGGTCGCCC GGGCAGGGTA   27540

TAACTCACCT GACAATCAGA GGGCGAGGTA TTCAGCTCAA CGACGAGTCG GTGAGCTCCT   27600

CGCTTGGTCT CCGTCCGGAC GGGACATTTC AGATCGGCGG CGCCGGCCGT CCTTCATTCA   27660

CGCCTCGTCA GGCAATCCTA ACTCTGCAGA CCTCGTCCTC TGAGCCGCGC TCTGGAGGCA   27720

TTGGAACTCT GCAATTTATT GAGGAGTTTG TGCCATCGGT CTACTTTAAC CCCTTCTCGG   27780
```

●

```
GACCTCCCGG CCACTATCCG GATCAATTTA TTCCTAACTT TGACGCGGTA AAGGACTCGG    27840
CGGACGGCTA CGACTGAATG TTAAGTGGAG AGGCAGAGCA ACTGCGCCTG AAACACCTGG    27900
TCCACTGTCG CCGCCACAAG TGCTTTGCCC GCGACTCCGG TGAGTTTTGC TACTTTGAAT    27960
TGCCCGAGGA TCATATCGAG GGCCCGGCGC ACGGCGTCCG GCTTACCGCC CAGGGAGAGC    28020
TTGCCCGTAG CCTGATTCGG GAGTTTACCC AGCGCCCCCT GCTAGTTGAG CGGGACAGGG    28080
GACCCTGTGT TCTCACTGTG ATTTGCAACT GTCCTAACCT TGGATTACAT CAAGATCTTT    28140
GTTGCCATCT CTGTGCTGAG TATAATAAAT ACAGAAATTA AAATATACTG GGGCTCCTAT    28200
CGCCATCCTG TAAACGCCAC CGTCTTCACC CGCCCAAGCA AACCAAGGCG AACCTTACCT    28260
GGTACTTTTA ACATCTCTCC CTCTGTGATT TACAACAGTT TCAACCCAGA CGGAGTGAGT    28320
CTACGAGAGA ACCTCTCCGA GCTCAGCTAC TCCATCAGAA AAAACACCAC CCTCCTTACC    28380
TGCCGGGAAC GTACGAGTGC GTCACCGGCC GCTGCACCAC ACCTACCGCC TGACCGTAAA    28440
CCAGACTTTT TCCGGACAGA CCTCAATAAC TCTGTTACCC AGAACAGGAG GTGAGCTTAG    28500
AAAACCCTTA GGGTATTAGG CCAAAGGCGC AGCTACTGTG GGGTTTATGA ACAATTCAAG    28560
CAACTCTACG GGCTATTCTA ATTCAGGTTT CTCTAGAATC GGGGTTGGGG TTATTCTCTG    28620
TCTTGTGATT CTCTTTATTC TTATACTAAC GCTTCTCTGC CTAAGGCTCG CCGCCTGCTG    28680
TGTGCACATT TGCATTTATT GTCAGCTTTT TAAACGCTGG GGTCGCCACC CAAGATGATT    28740
AGGTACATAA TCCTAGGTTT ACTCACCCTT GCGTCAGCCC ACGGTACCAC CCAAAAGGTG    28800
GATTTTAAGG AGCCAGCCTG TAATGTTACA TTCGCAGCTG AAGCTAATGA GTGCACCACT    28860
CTTATAAAAT GCACCACAGA ACATGAAAAG CTGCTTATTC GCCACAAAAA CAAAATTGGC    28920
AAGTATGCTG TTTATGCTAT TTGGCAGCCA GGTGACACTA CAGAGTATAA TGTTACAGTT    28980
TTCCAGGGTA AAAGTCATAA AACTTTTATG TATACTTTTC CATTTTATGA AATGTGCGAC    29040
ATTACCATGT ACATGAGCAA ACAGTATAAG TTGTGGCCCC CACAAAATTG TGTGGAAAAC    29100
ACTGGCACTT TCTGCTGCAC TGCTATGCTA ATTACAGTGC TCGCTTTGGT CTGTACCCTA    29160
CTCTATATTA AATACAAAAG CAGACGCAGC TTTATTGAGG AAAAGAAAAT GCCTTAATTT    29220
ACTAAGTTAC AAAGCTAATG TCACCACTAA CTGCTTTACT CGCTGCTTGC AAAACAAATT    29280
CAAAAAGTTA GCATTATAAT TAGAATAGGA TTTAAACCCC CCGGTCATTT CCTGCTCAAT    29340
ACCATTCCCC TGAACAATTG ACTCTATGTG GGATATGCTC CAGCGCTACA ACCTTGAAGT    29400
CAGGCTTCCT GGATGTCAGC ATCTGACTTT GGCCAGCACC TGTCCCGCGG ATTTGTTCCA    29460
GTCCAACTAC AGCGACCCAC CCTAACAGAG ATGACCAACA CAACCAACGC GGCCGCCGCT    29520
ACCGGACTTA CATCTACCAC AAATACACCC CAAGTTCTG CCTTTGTCAA TAACTGGGAT    29580
AACTTGGGCA TGTGGTGGTT CTCCATAGCG CTTATGTTTG TATGCCTTAT TATTATGTGG    29640
CTCATCTGCT GCCTAAAGCG CAAACGCGCC CGACCACCCA TCTATAGTCC CATCATTGTG    29700
CTACACCCAA ACAATGATGG AATCCATAGA TTGGACGGAC TGAAACACAT GTTCTTTTCT    29760
CTTACAGTAT GATTAAATGA GACATGATTC CTCGAGTTTT TATATTACTG ACCCTTGTTG    29820
CGCTTTTTTG TGCGTGCTCC ACATTGGCTG CGGTTTCTCA CATCGAAGTA GACTGCATTC    29880
```

```
CAGCCTTCAC AGTCTATTTG CTTTACGGAT TTGTCACCCT CACGCTCATC TGCAGCCTCA  29940
TCACTGTGGT CATCGCCTTT ATCCAGTGCA TTGACTGGGT CTGTGTGCGC TTTGCATATC  30000
TCAGACACCA TCCCCAGTAC AGGGACAGGA CTATAGCTGA GCTTCTTAGA ATTCTTTAAT  30060
TATGAAATTT ACTGTGACTT TTCTGCTGAT TATTTGCACC CTATCTGCGT TTTGTTCCCC  30120
GACCTCCAAG CCTCAAAGAC ATATATCATG CAGATTCACT CGTATATGGA ATATTCCAAG  30180
TTGCTACAAT GAAAAAAGCG ATCTTTCCGA AGCCTGGTTA TATGCAATCA TCTCTGTTAT  30240
GGTGTTCTGC AGTACCATCT TAGCCCTAGC TATATATCCC TACCTTGACA TTGGCTGGAA  30300
ACGAATAGAT GCCATGAACC ACCCAACTTT CCCCGCGCCC GCTATGCTTC CACTGCAACA  30360
AGTTGTTGCC GGCGGCTTTG TCCCAGCCAA TCAGCCTCGC CCCACTTCTC CCACCCCCAC  30420
TGAAATCAGC TACTTTAATC TAACAGGAGG AGATGACTGA CACCCTAGAT CTAGAAATGG  30480
ACGGAATTAT TACAGAGCAG CGCCTGCTAG AAAGACGCAG GGCAGCGGCC GAGCAACAGC  30540
GCATGAATCA AGAGCTCCAA GACATGGTTA ACTTGCACCA GTGCAAAAGG GGTATCTTTT  30600
GTCTGGTAAA GCAGGCCAAA GTCACCTACG ACAGTAATAC CACCGGACAC CGCCTTAGCT  30660
ACAAGTTGCC AACCAAGCGT CAGAAATTGG TGGTCATGGT GGGAGAAAAG CCCATTACCA  30720
TAACTCAGCA CTCGGTAGAA ACCGAAGGCT GCATTCACTC ACCTTGTCAA GGACCTGAGG  30780
ATCTCTGCAC CCTTATTAAG ACCCTGTGCG GTCTCAAAGA TCTTATTCCC TTTAACTAAT  30840
AAAAAAAAAT AATAAAGCAT CACTTACTTA AAATCAGTTA GCAAATTTCT GTCCAGTTTA  30900
TTCAGCAGCA CCTCCTTGCC CTCCTCCCAG CTCTGGTATT GCAGCTTCCT CCTGGCTGCA  30960
AACTTTCTCC ACAATCTAAA TGGAATGTCA GTTTCCTCCT GTTCCTGTCC ATCCGCACCC  31020
ACTATCTTCA TGTTGTTGCA GATGAAGCGC GCAAGACCGT CTGAAGATAC CTTCAACCCC  31080
GTGTATCCAT ATGACACGGA AACCGGTCCT CCAACTGTGC CTTTTCTTAC TCCTCCCTTT  31140
GTATCCCCCA ATGGGTTTCA AGAGAGTCCC CCTGGGGTAC TCTCTTTGCG CCTATCCGAA  31200
CCTCTAGTTA CCTCCAATGG CATGCTTGCG CTCAAAATGG CAACGGCCT CTCTCTGGAC  31260
GAGGCCGGCA ACCTTACCTC CCAAAATGTA ACCACTGTGA GCCCACCTCT CAAAAAAACC  31320
AAGTCAAACA TAAACCTGGA AATATCTGCA CCCCTCACAG TTACCTCAGA AGCCCTAACT  31380
GTGGCTGCCG CCGCACCTCT AATGGTCGCG GGCAACACAC TCACCATGCA ATCACAGGCC  31440
CCGCTAACCG TGCACGACTC CAAACTTAGC ATTGCCACCC AAGGACCCCT CACAGTGTCA  31500
GAAGGAAAGC TAGCCCTGCA AACATCAGGC CCCCTCACCA CCACCGATAG CAGTACCCTT  31560
ACTATCACTG CCTCACCCCC TCTAACTACT GCCACTGGTA GCTTGGGCAT TGACTTGAAA  31620
GAGCCCATTT ATACACAAAA TGGAAAACTA GGACTAAAGT ACGGGGCTCC TTTGCATGTA  31680
ACAGACGACC TAAACACTTT GACCGTAGCA ACTGGTCCAG GTGTGACTAT TAATAATACT  31740
TCCTTGCAAA CTAAAGTTAC TGGAGCCTTG GGTTTTGATT CACAAGGCAA TATGCAACTT  31800
AATGTAGCAG GAGGACTAAG GATTGATTCT CAAAACAGAC GCCTTATACT TGATGTTAGT  31860
TATCCGTTTG ATGCTCAAAA CCAACTAAAT CTAAGACTAG GACAGGGCCC TCTTTTTATA  31920
AACTCAGCCC ACAACTTGGA TATTAACTAC AACAAAGGCC TTTACTTGTT TACAGCTTCA  31980
```

```
AACAATTCCA AAAAGCTTGA GGTTAACCTA AGCACTGCCA AGGGGTTGAT GTTTGACGCT    32040
ACAGCCATAG CCATTAATGC AGGAGATGGG CTTGAATTTG GTTCACCTAA TGCACCAAAC    32100
ACAAATCCCC TCAAAACAAA AATTGGCCAT GGCCTAGAAT TTGATTCAAA CAAGGCTATG    32160
GTTCCTAAAC TAGGAACTGG CCTTAGTTTT GACAGCACAG GTGCCATTAC AGTAGGAAAC    32220
AAAAATAATG ATAAGCTAAC TTTGTGGACC ACACCAGCTC CATCTCCTAA CTGTAGACTA    32280
AATGCAGAGA AAGATGCTAA ACTCACTTTG GTCTTAACAA AATGTGGCAG TCAAATACTT    32340
GCTACAGTTT CAGTTTTGGC TGTTAAAGGC AGTTTGGCTC CAATATCTGG AACAGTTCAA    32400
AGTGCTCATC TTATTATAAG ATTTGACGAA AATGGAGTGC TACTAAACAA TTCCTTCCTG    32460
GACCCAGAAT ATTGGAACTT TAGAAATGGA GATCTTACTG AAGGCACAGC CTATACAAAC    32520
GCTGTTGGAT TTATGCCTAA CCTATCAGCT TATCCAAAAT CTCACGGTAA AACTGCCAAA    32580
AGTAACATTG TCAGTCAAGT TTACTTAAAC GGAGACAAAA CTAAACCTGT AACACTAACC    32640
ATTACACTAA ACGGTACACA GGAAACAGGA GACACAACTC CAAGTGCATA CTCTATGTCA    32700
TTTTCATGGG ACTGGTCTGG CCACAACTAC ATTAATGAAA TATTTGCCAC ATCCTCTTAC    32760
ACTTTTTCAT ACATTGCCCA AGAATAAAGA ATCGTTTGTG TTATGTTTCA ACGTGTTTAT    32820
TTTTCAATTG CAGAAAATTT CAAGTCATTT TTCATTCAGT AGTATAGCCC CACCACCACA    32880
TAGCTTATAC AGATCACCGT ACCTTAATCA AACTCACAGA ACCCTAGTAT TCAACCTGCC    32940
ACCTCCCTCC CAACACACAG AGTACACAGT CCTTTCTCCC CGGCTGGCCT TAAAAAGCAT    33000
CATATCATGG GTAACAGACA TATTCTTAGG TGTTATATTC CACACGGTTT CCTGTCGAGC    33060
CAAACGCTCA TCAGTGATAT TAATAAACTC CCCGGGCAGC TCACTTAAGT TCATGTCGCT    33120
GTCCAGCTGC TGAGCCACAG GCTGCTGTCC AACTTGCGGT TGCTTAACGG GCGGCGAAGG    33180
AGAAGTCCAC GCCTACATGG GGGTAGAGTC ATAATCGTGC ATCAGGATAG GGCGGTGGTG    33240
CTGCAGCAGC GCGCGAATAA ACTGCTGCCG CCGCCGCTCC GTCCTGCAGG AATACAACAT    33300
GGCAGTGGTC TCCTCAGCGA TGATTCGCAC CGCCCGCAGC ATAAGGCGCC TTGTCCTCCG    33360
GGCACAGCAG CGCACCCTGA TCTCACTTAA ATCAGCACAG TAACTGCAGC ACAGCACCAC    33420
AATATTGTTC AAAATCCCAC AGTGCAAGGC GCTGTATCCA AAGCTCATGG CGGGGACCAC    33480
AGAACCCACG TGGCCATCAT ACCACAAGCG CAGGTAGATT AAGTGGCGAC CCCTCATAAA    33540
CACGCTGGAC ATAAACATTA CCTCTTTTGG CATGTTGTAA TTCACCACCT CCCGGTACCA    33600
TATAAACCTC TGATTAAACA TGGCGCCATC CACCACCATC CTAAACCAGC TGGCCAAAAC    33660
CTGCCCGCCG GCTATACACT GCAGGGAACC GGGACTGGAA CAATGACAGT GGAGAGCCCA    33720
GGACTCGTAA CCATGGATCA TCATGCTCGT CATGATATCA ATGTTGGCAC AACACAGGCA    33780
CACGTGCATA CACTTCCTCA GGATTACAAG CTCCTCCCGC GTTAGAACCA TATCCCAGGG    33840
AACAACCCAT TCCTGAATCA GCGTAAATCC CACACTGCAG GGAAGACCTC GCACGTAACT    33900
CACGTTGTGC ATTGTCAAAG TGTTACATTC GGGCAGCAGC GGATGATCCT CCAGTATGGT    33960
AGCGCGGGTT TCTGTCTCAA AAGGAGGTAG ACGATCCCTA CTGTACGGAG TGCGCCGAGA    34020
CAACCGAGAT CGTGTTGGTC GTAGTGTCAT GCCAAATGGA ACGCCGGACG TAGTCATATT    34080
```

```
TCCTGAAGCA AAACCAGGTG CGGGCGTGAC AAACAGATCT GCGTCTCCGG TCTCGCCGCT    34140

TAGATCGCTC TGTGTAGTAG TTGTAGTATA TCCACTCTCT CAAAGCATCC AGGCGCCCCC    34200

TGGCTTCGGG TTCTATGTAA ACTCCTTCAT GCGCCGCTGC CCTGATAACA TCCACCACCG    34260

CAGAATAAGC CACACCCAGC CAACCTACAC ATTCGTTCTG CGAGTCACAC ACGGGAGGAG    34320

CGGGAAGAGC TGGAAGAACC ATGTTTTTTT TTTATTCCA AAAGATTATC CAAAACCTCA     34380

AAATGAAGAT CTATTAAGTG AACGCGCTCC CCTCCGGTGG CGTGGTCAAA CTCTACAGCC    34440

AAAGAACAGA TAATGGCATT TGTAAGATGT TGCACAATGG CTTCCAAAAG GCAAACGGCC    34500

CTCACGTCCA AGTGGACGTA AAGGCTAAAC CCTTCAGGGT GAATCTCCTC TATAAACATT    34560

CCAGCACCTT CAACCATGCC CAAATAATTC TCATCTCGCC ACCTTCTCAA TATATCTCTA    34620

AGCAAATCCC GAATATTAAG TCCGGCCATT GTAAAAATCT GCTCCAGAGC GCCCTCCACC    34680

TTCAGCCTCA AGCAGCGAAT CATGATTGCA AAAATTCAGG TTCCTCACAG ACCTGTATAA    34740

GATTCAAAAG CGGAACATTA ACAAAAATAC CGCGATCCCG TAGGTCCCTT CGCAGGGCCA    34800

GCTGAACATA ATCGTGCAGG TCTGCACGGA CCAGCGCGGC CACTTCCCCG CCAGGAACCT    34860

TGACAAAAGA ACCCACACTG ATTATGACAC GCATACTCGG AGCTATGCTA ACCAGCGTAG    34920

CCCCGATGTA AGCTTTGTTG CATGGGCGGC GATATAAAAT GCAAGGTGCT GCTCAAAAAA    34980

TCAGGCAAAG CCTCGCGCAA AAAAGAAAGC ACATCGTAGT CATGCTCATG CAGATAAAGG    35040

CAGGTAAGCT CCGGAACCAC CACAGAAAAA GACACCATTT TTCTCTCAAA CATGTCTGCG    35100

GGTTTCTGCA TAAACACAAA ATAAAATAAC AAAAAAACAT TTAAACATTA GAAGCCTGTC    35160

TTACAACAGG AAAAACAACC CTTATAAGCA TAAGACGGAC TACGGCCATG CCGGCGTGAC    35220

CGTAAAAAAA CTGGTCACCG TGATTAAAAA GCACCACCGA CAGCTCCTCG GTCATGTCCG    35280

GAGTCATAAT GTAAGACTCG GTAAACACAT CAGGTTGATT CATCGGTCAG TGCTAAAAAG    35340

CGACCGAAAT AGCCCGGGGG AATACATACC CGCAGGCGTA GAGACAACAT TACAGCCCCC    35400

ATAGGAGGTA TAACAAAATT AATAGGAGAG AAAAACACAT AAACACCTGA AAAACCCTCC    35460

TGCCTAGGCA AAATAGCACC CTCCCGCTCC AGAACAACAT ACAGCGCTTC ACAGCGGCAG    35520

CCTAACAGTC AGCCTTACCA GTAAAAAGA AAACCTATTA AAAAAACACC ACTCGACACG     35580

GCACCAGCTC AATCAGTCAC AGTGTAAAAA AGGGCCAAGT GCAGAGCGAG TATATATAGG    35640

ACTAAAAAAT GACGTAACGG TTAAAGTCCA CAAAAAACAC CCAGAAAACC GCACGCGAAC    35700

CTACGCCCAG AAACGAAAGC CAAAAAACCC ACAACTTCCT CAAATCGTCA CTTCCGTTTT    35760

CCCACGTTAC GTAACTTCCC ATTTTAAGAA AACTACAATT CCCAACACAT ACAAGTTACT    35820

CCGCCCTAAA ACCTACGTCA CCCGCCCCGT TCCCACGCCC CGCGCCACGT CACAAACTCC    35880

ACCCCCTCAT TATCATATTG GCTTCAATCC AAAATAAGGT ATATTATTGA TGATG         35935
```

(2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 25 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

```
    (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION: /desc = "DNA"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
```

TTCATTTTAT GTTTCAGGTT CAGGG   25

(2) INFORMATION FOR SEQ ID NO:3:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION: /desc = "DNA"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
```

TTACCGCCAC ACTCGCAGGG   20

(2) INFORMATION FOR SEQ ID NO:4:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34303 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
         (A) DESCRIPTION: /desc = "DNA"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
```

```
TTATTTTGGA TTGAAGCCAA TATGATAATG AGGGGGTGGA GTTTGTGACG TGGCGCGGGG      60

CGTGGGAACG GGGCGGGTGA CGTAGTAGTG TGGCGGAAGT GTGATGTTGC AAGTGTGGCG     120

GAACACATGT AAGCGACGGA TGTGGCAAAA GTGACGTTTT TGGTGTGCGC CGGATCCACA     180

GGACGGGTGT GGTCGCCATG ATCGCGTAGT CGATAGTGGC TCCAAGTAGC GAAGCGAGCA     240

GGACTGGGCG GCGGCCAAAG CGGTCGGACA GTGCTCCGAG AACGGGTGCG CATAGAAATT     300

GCATCAACGC ATATAGCGCT AGCAGCACGC CATAGTGACT GGCGATGCTG TCGGAATGGA     360

CGATATCCCG CAAGAGGCCC GGCAGTACCG GCATAACCAA GCCTATGCCT ACAGCATCCA     420

GGGTGACGGT GCCGAGGATG ACGATGAGCG CATTGTTAGA TTTCATACAC GGTGCCTGAC     480

TGCGTTAGCA ATTTAACTGT GATAAACTAC CGCATTAAAG CTTATCGATG ATAAGCTGTC     540

AAACATGAGA ATTCTTGAAG ACGAAAGGGC CTCGTGATAC GCCTATTTTT ATAGGTTAAT     600

GTCATGATAA TAATGGTTTC TTAGACGTCA GGTGGCACTT TTCGGGGAAA TGTGCGCGGA     660

ACCCCTATTT GTTTATTTTT CTAAATACAT TCAAATATGT ATCCGCTCAT GAGACAATAA     720

CCCTGATAAA TGCTTCAATA ATATTGAAAA AGGAAGAGTA TGAGTATTCA ACATTTCCGT     780

GTCGCCCTTA TTCCCTTTTT TGCGGCATTT TGCCTTCCTG TTTTTGCTCA CCCAGAAACG     840

CTGGTGAAAG TAAAAGATGC TGAAGATCAG TTGGGTGCAC GAGTGGGTTA CATCGAACTG     900

GATCTCAACA GCGGTAAGAT CCTTGAGAGT TTTCGCCCCG AAGAACGTTT TCCAATGATG     960

AGCACTTTTA AAGTTCTGCT ATGTGGCGCG GTATTATCCC GTGTTGACGC CGGGCAAGAG    1020

CAACTCGGTC GCCGCATACA CTATTCTCAG AATGACTTGG TTGAGTACTC ACCAGTCACA    1080
```

```
GAAAAGCATC TTACGGATGG CATGACAGTA AGAGAATTAT GCAGTGCTGC CATAACCATG    1140

AGTGATAACA CTGCGGCCAA CTTACTTCTG ACAACGATCG GAGGACCGAA GGAGCTAACC    1200

GCTTTTTTGC ACAACATGGG GGATCATGTA ACTCGCCTTG ATCGTTGGGA ACCGGAGCTG    1260

AATGAAGCCA TACCAAACGA CGAGCGTGAC ACCACGATGC CTGCAGCAAT GGCAACAACG    1320

TTGCGCAAAC TATTAACTGG CGAACTACTT ACTCTAGCTT CCCGGCAACA ATTAATAGAC    1380

TGGATGGAGG CGGATAAAGT TGCAGGACCA CTTCTGCGCT CGGCCCTTCC GGCTGGCTGG    1440

TTTATTGCTG ATAAATCTGG AGCCGGTGAG CGTGGGTCTC GCGGTATCAT TGCAGCACTG    1500

GGGCCAGATG GTAAGCCCTC CCGTATCGTA GTTATCTACA CGACGGGGAG TCAGGCAACT    1560

ATGGATGAAC GAAATAGACA GATCGCTGAG ATAGGTGCCT CACTGATTAA GCATTGGTAA    1620

CTGTCAGACC AAGTTTACTC ATATATACTT TAGATTGATT TAAAACTTCA TTTTTAATTT    1680

AAAAGGATCT AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC TTAACGTGAG    1740

TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA AAGGATCTTC TTGAGATCCT    1800

TTTTTTCTGC GCGTAATCTG CTGCTTGCAA ACAAAAAAAC CACCGCTACC AGCGGTGGTT    1860

TGTTTGCCGG ATCAAGAGCT ACCAACTCTT TTTCCGAAGG TAACTGGCTT CAGCAGAGCG    1920

CAGATACCAA ATACTGTCCT TCTAGTGTAG CCGTAGTTAG GCCACCACTT CAAGAACTCT    1980

GTAGCACCGC CTACATACCT CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC TGCCAGTGGC    2040

GATAAGTCGT GTCTTACCGG GTTGGACTCA AGACGATAGT TACCGGATAA GGCGCAGCGG    2100

TCGGGCTGAA CGGGGGGTTC GTGCACACAG CCCAGCTTGG AGCGAACGAC CTACACCGAA    2160

CTGAGATACC TACAGCGTGA GCATTGAGAA AGCGCCACGC TTCCCGAAGG GAGAAAGGCG    2220

GACAGGTATC CGGTAAGCGG CAGGGTCGGA ACAGGAGAGC GCACGAGGGA GCTTCCAGGG    2280

GGAAACGCCT GGTATCTTTA TAGTCCTGTC GGGTTTCGCC ACCTCTGACT TGAGCGTCGA    2340

TTTTTGTGAT GCTCGTCAGG GGGGCGGAGC CTATGGAAAA ACGCCAGCAA CGCGGCCTTT    2400

TTACGGTTCC TGGCCTTTTG CTGGCCTTTT GCTCACATGT TCTTTCCTGC GTTATCCCCT    2460

GATTCTGTGG ATAACCGTAT TACCGCCTTT GAGTGAGCTG ATACCGCTCG CCGCAGCCGA    2520

ACGACCGAGC GCAGCGAGTC AGTGAGCGAG GAAGCGGAAG AGCGCCTGAT GCGGTATTTT    2580

CTCCTTACGC ATCTGTGCGG TATTTCACAC CGCATATGGT GCACTCTCAG TACAATCTGC    2640

TCTGATGCCG CATAGTTAAG CCAGTATACA CTCCGCTATC GCTACGTGAC TGGGTCATGG    2700

CTGCGCCCCG ACACCCGCCA ACACCCGCTG ACGCGCCCTG ACGGGCTTGT CTGCTCCCGG    2760

CATCCGCTTA CAGACAAGCT GTGACCGTCT CCGGGAGCTG CATGTGTCAG AGGTTTTCAC    2820

CGTCATCACC GAAACGCGCG AGGCAGTCTA GACAATAGTA GTACGGATAG CTGTGACTCC    2880

GGTCCTTCTA ACACACCTCC TGAGATACAC CCGGTGGTCC CGCTGTGCCC CATTAAACCA    2940

GTTGCCGTGA GAGTTGGTGG GCGTCGCCAG GCTGTGGAAT GTATCGAGGA CTTGCTTAAC    3000

GAGCCTGGGC AACCTTTGGA CTTGAGCTGT AAACGCCCCA GGCCATAAGG TGTAAACCTG    3060

TGATTGCGTG TGTGGTTAAC GCCTTTGTTT GCTGAATGAG TTGATGTAAG TTTAATAAAG    3120

GGTGAGATAA TGTTTAACTT GCATGGCGTG TTAAATGGGG CGGGGCTTAA AGGGTATATA    3180
```

```
ATGCGCCGTG GGCTAATCTT GGTTACATCT GACCTCATGG AGGCTTGGGA GTGTTTGGAA      3240

GATTTTTCTG CTGTGCGTAA CTTGCTGGAA CAGAGCTCTA ACAGTACCTC TTGGTTTTGG      3300

AGGTTTCTGT GGGGCTCATC CCAGGCAAAG TTAGTCTGCA GAATTAAGGA GGATTACAAG      3360

TGGGAATTTG AAGAGCTTTT GAAATCCTGT GGTGAGCTGT TTGATTCTTT GAATCTGGGT      3420

CACCAGGCGC TTTTCCAAGA GAAGGTCATC AAGACTTTGG ATTTTTCCAC ACCGGGGCGC      3480

GCTGCGGCTG CTGTTGCTTT TTTGAGTTTT ATAAAGGATA AATGGAGCGA AGAAACCCAT      3540

CTGAGCGGGG GGTACCTGCT GGATTTTCTG CCATGCATC TGTGGAGAGC GGTTGTGAGA      3600

CACAAGAATC GCCTGCTACT GTTGTCTTCC GTCCGCCCGG CGATAATACC GACGGAGGAG      3660

CAGCAGCAGC AGCAGGAGGA AGCCAGGCGG CGGCGGCAGG AGCAGAGCCC ATGGAACCCG      3720

AGAGCCGGCC TGGACCCTCG GGAATGAATG TTGTACAGGT GGCTGAACTG TATCCAGAAC      3780

TGAGACGCAT TTTGACAATT ACAGAGGATG GGCAGGGGCT AAAGGGGGTA AAGAGGGAGC      3840

GGGGGGCTTG TGAGGCTACA GAGGAGGCTA GGAATCTAGC TTTTAGCTTA ATGACCAGAC      3900

ACCGTCCTGA GTGTATTACT TTTCAACAGA TCAAGGATAA TTGCGCTAAT GAGCTTGATC      3960

TGCTGGCGCA GAAGTATTCC ATAGAGCAGC TGACCACTTA CTGGCTGCAG CCAGGGGATG      4020

ATTTTGAGGA GGCTATTAGG GTATATGCAA AGGTGGCACT TAGGCCAGAT TGCAAGTACA      4080

AGATCAGCAA ACTTGTAAAT ATCAGGAATT GTTGCTACAT TTCTGGGAAC GGGGCCGAGG      4140

TGGAGATAGA TACGGAGGAT AGGGTGGCCT TTAGATGTAG CATGATAAAT ATGTGGCCGG      4200

GGGTGCTTGG CATGGACGGG GTGGTTATTA TGAATGTAAG GTTTACTGGC CCCAATTTTA      4260

GCGGTACGGT TTTCCTGGCC AATACCAACC TTATCCTACA CGGTGTAAGC TTCTATGGGT      4320

TTAACAATAC CTGTGTGGAA GCCTGGACCG ATGTAAGGGT TCGGGGCTGT GCCTTTTACT      4380

GCTGCTGGAA GGGGGTGGTG TGTCGCCCCA AAAGCAGGGC TTCAATTAAG AAATGCCTCT      4440

TTGAAAGGTG TACCTTGGGT ATCCTGTCTG AGGGTAACTC CAGGGTGCGC CACAATGTGG      4500

CCTCCGACTG TGGTTGCTTC ATGCTAGTGA AAAGCGTGGC TGTGATTAAG CATAACATGG      4560

TATGTGGCAA CTGCGAGGAC AGGGCCTCTC AGATGCTGAC CTGCTCGGAC GGCAACTGTC      4620

ACCTGCTGAA GACCATTCAC GTAGCCAGCC ACTCTCGCAA GGCCTGGCCA GTGTTTGAGC      4680

ATAACATACT GACCCGCTGT TCCTTGCATT TGGGTAACAG GAGGGGGGTG TTCCTACCTT      4740

ACCAATGCAA TTTGAGTCAC ACTAAGATAT TGCTTGAGCC CGAGAGCATG TCCAAGGTGA      4800

ACCTGAACGG GGTGTTTGAC ATGACCATGA AGATCTGGAA GGTGCTGAGG TACGATGAGA      4860

CCCGCACCAG GTGCAGACCC TGCGAGTGTG GCGGTAAACA TATTAGGAAC CAGCCTGTGA      4920

TGCTGGATGT GACCGAGGAG CTGAGGCCCG ATCACTTGGT GCTGGCCTGC ACCCGCGCTG      4980

AGTTTGGCTC TAGCGATGAA GATACAGATT GAGGTACTGA AATGTGTGGG CGTGGCTTAA      5040

GGGTGGGAAA GAATATATAA GGTGGGGGTC TTATGTAGTT TTGTATCTGT TTTGCAGCAG      5100

CCGCCGCCGC CATGAGCACC AACTCGTTTG ATGGAAGCAT TGTGAGCTCA TATTTGACAA      5160

CGCGCATGCC CCCATGGGCC GGGGTGCGTC AGAATGTGAT GGGCTCCAGC ATTGATGGTC      5220

GCCCCGTCCT GCCCGCAAAC TCTACTACCT TGACCTACGA GACCGTGTCT GGAACGCCGT      5280
```

```
TGGAGACTGC AGCCTCCGCC GCCGCTTCAG CCGCTGCAGC CACCGCCCGC GGGATTGTGA    5340

CTGACTTTGC TTTCCTGAGC CCGCTTGCAA GCAGTGCAGC TTCCCGTTCA TCCGCCCGCG    5400

ATGACAAGTT GACGGCTCTT TTGGCACAAT TGGATTCTTT GACCCGGGAA CTTAATGTCG    5460

TTTCTCAGCA GCTGTTGGAT CTGCGCCAGC AGGTTTCTGC CCTGAAGGCT TCCTCCCCTC    5520

CCAATGCGGT TTAAAACATA AATAAAAAAC CAGACTCTGT TTGGATTTGG ATCAAGCAAG    5580

TGTCTTGCTG TCTTTATTTA GGGGTTTTGC GCGCGCGGTA GGCCCGGGAC CAGCGGTCTC    5640

GGTCGTTGAG GGTCCTGTGT ATTTTTTCCA GGACGTGGTA AAGGTGACTC TGGATGTTCA    5700

GATACATGGG CATAAGCCCG TCTCTGGGGT GGAGGTAGCA CCACTGCAGA GCTTCATGCT    5760

GCGGGGTGGT GTTGTAGATG ATCCAGTCGT AGCAGGAGCG CTGGGCGTGG TGCCTAAAAA    5820

TGTCTTTCAG TAGCAAGCTG ATTGCCAGGG GCAGGCCCTT GGTGTAAGTG TTTACAAAGC    5880

GGTTAAGCTG GGATGGGTGC ATACGTGGGG ATATGAGATG CATCTTGGAC TGTATTTTTA    5940

GGTTGGCTAT GTTCCCAGCC ATATCCCTCC GGGGATTCAT GTTGTGCAGA ACCACCAGCA    6000

CAGTGTATCC GGTGCACTTG GGAAATTTGT CATGTAGCTT AGAAGGAAAT GCGTGGAAGA    6060

ACTTGGAGAC GCCCTTGTGA CCTCCAAGAT TTTCCATGCA TTCGTCCATA ATGATGGCAA    6120

TGGGCCCACG GCGGCGGCC TGGGCGAAGA TATTTCTGGG ATCACTAACG TCATAGTTGT    6180

GTTCCAGGAT GAGATCGTCA TAGGCCATTT TTACAAAGCG CGGGCGGAGG GTGCCAGACT    6240

GCGGTATAAT GGTTCCATCC GGCCCAGGGG CGTAGTTACC CTCACAGATT TGCATTTCCC    6300

ACGCTTTGAG TTCAGATGGG GGGATCATGT CTACCTGCGG GGCGATGAAG AAAACGGTTT    6360

CCGGGGTAGG GGAGATCAGC TGGGAAGAAA GCAGGTTCCT GAGCAGCTGC GACTTACCGC    6420

AGCCGGTGGG CCCGTAAATC ACACCTATTA CCGGGTGCAA CTGGTAGTTA AGAGAGCTGC    6480

AGCTGCCGTC ATCCCTGAGC AGGGGGGCCA CTTCGTTAAG CATGTCCCTG ACTCGCATGT    6540

TTTCCCTGAC CAAATCCGCC AGAAGGCGCT CGCCGCCCAG CGATAGCAGT TCTTGCAAGG    6600

AAGCAAAGTT TTTCAACGGT TTGAGACCGT CCGCCGTAGG CATGCTTTTG AGCGTTTGAC    6660

CAAGCAGTTC CAGGCGGTCC CACAGCTCGG TCACCTGCTC TACGGCATCT CGATCCAGCA    6720

TATCTCCTCG TTTCGCGGGT TGGGGCGGCT TTCGCTGTAC GGCAGTAGTC GGTGCTCGTC    6780

CAGACGGGCC AGGGTCATGT CTTTCCACGG CGCAGGGTC CTCGTCAGCG TAGTCTGGGT    6840

CACGGTGAAG GGGTGCGCTC CGGGCTGCGC GCTGGCCAGG GTGCGCTTGA GGCTGGTCCT    6900

GCTGGTGCTG AAGCGCTGCC GGTCTTCGCC CTGCGCGTCG GCCAGGTAGC ATTTGACCAT    6960

GGTGTCATAG TCCAGCCCCT CCGCGGCGTG GCCCTTGGCG CGCAGCTTGC CCTTGGAGGA    7020

GGCGCCGCAC GAGGGGCAGT GCAGACTTTT GAGGGCGTAG AGCTTGGGCG CGAGAAATAC    7080

CGATTCCGGG GAGTAGGCAT CCGCGCCGCA GGCCCCGCAG ACGGTCTCGC ATTCCACGAG    7140

CCAGGTGAGC TCTGGCCGTT CGGGGTCAAA AACCAGGTTT CCCCCATGCT TTTTGATGCG    7200

TTTCTTACCT CTGGTTTCCA TGAGCCGGTG TCCACGCTCG GTGACGAAAA GGCTGTCCGT    7260

GTCCCCGTAT ACAGACTTGA GAGGCCTGTC CTCGAGCGGT GTTCCGCGGT CCTCCTCGTA    7320

TAGAAACTCG GACCACTCTG AGACAAAGGC TCGCGTCCAG GCCAGCACGA AGGAGGCTAA    7380
```

- 90 -

```
GTGGGAGGGG TAGCGGTCGT TGTCCACTAG GGGGTCCACT CGCTCCAGGG TGTGAAGACA    7440

CATGTCGCCC TCTTCGGCAT CAAGGAAGGT GATTGGTTTG TAGGTGTAGG CCACGTGACC    7500

GGGTGTTCCT GAAGGGGGGC TATAAAAGGG GGTGGGGGCG CGTTCGTCCT CACTCTCTTC    7560

CGCATCGCTG TCTGCGAGGG CCAGCTGTTG GGGTGAGTAC TCCCTCTGAA AAGCGGGCAT    7620

GACTTCTGCG CTAAGATTGT CAGTTTCCAA AAACGAGGAG GATTTGATAT TCACCTGGCC    7680

CGCGGTGATG CCTTTGAGGG TGGCCGCATC CATCTGGTCA GAAAAGACAA TCTTTTTGTT    7740

GTCAAGCTTG GTGGCAAACG ACCCGTAGAG GGCGTTGGAC AGCAACTTGG CGATGGAGCG    7800

CAGGGTTTGG TTTTTGTCGC GATCGGCGCG CTCCTTGGCC GCGATGTTTA GCTGCACGTA    7860

TTCGCGCGCA ACGCACCGCC ATTCGGGAAA GACGGTGGTG CGCTCGTCGG GCACCAGGTG    7920

CACGCGCCAA CCGCGGTTGT GCAGGGTGAC AAGGTCAACG CTGGTGGCTA CCTCTCCGCG    7980

TAGGCGCTCG TTGGTCCAGC AGAGGCGGCC GCCCTTGCGC GAGCAGAATG GCGGTAGGGG    8040

GTCTAGCTGC GTCTCGTCCG GGGGGTCTGC GTCCACGGTA AAGACCCCGG GCAGCAGGCG    8100

CGCGTCGAAG TAGTCTATCT TGCATCCTTG CAAGTCTAGC GCCTGCTGCC ATGCGCGGGC    8160

GGCAAGCGCG CGCTCGTATG GGTTGAGTGG GGGACCCCAT GGCATGGGGT GGGTGAGCGC    8220

GGAGGCGTAC ATGCCGCAAA TGTCGTAAAC GTAGAGGGGC TCTCTGAGTA TTCCAAGATA    8280

TGTAGGGTAG CATCTTCCAC CGCGGATGCT GGCGCGCACG TAATCGTATA GTTCGTGCGA    8340

GGGAGCGAGG AGGTCGGGAC CGAGGTTGCT ACGGGCGGGC TGCTCTGCTC GGAAGACTAT    8400

CTGCCTGAAG ATGGCATGTG AGTTGGATGA TATGGTTGGA CGCTGGAAGA CGTTGAAGCT    8460

GGCGTCTGTG AGACCTACCG CGTCACGCAC GAAGGAGGCG TAGGAGTCGC GCAGCTTGTT    8520

GACCAGCTCG GCGGTGACCT GCACGTCTAG GGCGCAGTAG TCCAGGGTTT CCTTGATGAT    8580

GTCATACTTA TCCTGTCCCT TTTTTTTCCA CAGCTCGCGG TTGAGGACAA ACTCTTCGCG    8640

GTCTTTCCAG TACTCTTGGA TCGGAAACCC GTCGGCCTCC GAACGGTAAG AGCCTAGCAT    8700

GTAGAACTGG TTGACGGCCT GGTAGGCGCA GCATCCCTTT TCTACGGGTA GCGCGTATGC    8760

CTGCGCGGCC TTCCGGAGCG AGGTGTGGGT GAGCGCAAAG GTGTCCCTGA CCATGACTTT    8820

GAGGTACTGG TATTTGAAGT CAGTGTCGTC GCATCCGCCC TGCTCCCAGA GCAAAAAGTC    8880

CGTGCGCTTT TTGGAACGCG GATTTGGCAG GGCGAAGGTG ACATCGTTGA AGAGTATCTT    8940

TCCCGCGCGA GGCATAAAGT TGCGTGTGAT GCGGAAGGGT CCCGGCACCT CGGAACGGTT    9000

GTTAATTACC TGGGCGGCGA GCACGATCTC GTCAAAGCCG TTGATGTTGT GGCCCACAAT    9060

GTAAAGTTCC AAGAAGCGCG GGATGCCCTT GATGGAAGGC AATTTTTTAA GTTCCTCGTA    9120

GGTGAGCTCT TCAGGGGAGC TGAGCCCGTG CTCTGAAAGG GCCCAGTCTG CAAGATGAGG    9180

GTTGGAAGCG ACGAATGAGC TCCACAGGTC ACGGGCCATT AGCATTTGCA GGTGGTCGCG    9240

AAAGGTCCTA AACTGGCGAC CTATGGCCAT TTTTTCTGGG GTGATGCAGT AGAAGGTAAG    9300

CGGGTCTTGT TCCCAGCGGT CCCATCCAAG GTTCGCGGCT AGGTCTCGCG CGGCAGTCAC    9360

TAGAGGCTCA TCTCCGCCGA ACTTCATGAC CAGCATGAAG GGCACGAGCT GCTTCCCAAA    9420

GGCCCCCATC CAAGTATAGG TCTCTACATC GTAGGTGACA AAGAGACGCT CGGTGCGAGG    9480
```

```
ATGCGAGCCG ATCGGGAAGA ACTGGATCTC CCGCCACCAA TTGGAGGAGT GGCTATTGAT      9540

GTGGTGAAAG TAGAAGTCCC TGCGACGGGC CGAACACTCG TGCTGGCTTT TGTAAAAACG       9600

TGCGCAGTAC TGGCAGCGGT GCACGGGCTG TACATCCTGC ACGAGGTTGA CCTGACGACC      9660

GCGCACAAGG AAGCAGAGTG GGAATTTGAG CCCCTCGCCT GGCGGGTTTG GCTGGTGGTC      9720

TTCTACTTCG GCTGCTTGTC CTTGACCGTC TGGCTGCTCG AGGGGAGTTA CGGTGGATCG      9780

GACCACCACG CCGCGCGAGC CCAAAGTCCA GATGTCCGCG CGCGGCGGTC GGAGCTTGAT      9840

GACAACATCG CGCAGATGGG AGCTGTCCAT GGTCTGGAGC TCCCGCGGCG TCAGGTCAGG      9900

CGGGAGCTCC TGCAGGTTTA CCTCGCATAG ACGGGTCAGG GCGCGGGCTA GATCCAGGTG      9960

ATACCTAATT TCCAGGGGCT GGTTGGTGGC GGCGTCGATG GCTTGCAAGA GGCCGCATCC     10020

CCGCGGCGCG ACTACGGTAC CGCGCGGCGG GCGGTGGGCC GCGGGGGTGT CCTTGGATGA     10080

TGCATCTAAA AGCGGTGACG CGGGCGAGCC CCCGGAGGTA GGGGGGGCTC CGGACCCGCC     10140

GGGAGAGGGG GCAGGGGCAC GTCGGCGCCG CGCGCGGGCA GGAGCTGGTG CTGCGCGCGT     10200

AGGTTGCTGG CGAACGCGAC GACGCGGCGG TTGATCTCCT GAATCTGGCG CCTCTGCGTG     10260

AAGACGACGG GCCCGGTGAG CTTGAGCCTG AAAGAGAGTT CGACAGAATC AATTTCGGTG     10320

TCGTTGACGG CGGCCTGGCG CAAAATCTCC TGCACGTCTC CTGAGTTGTC TTGATAGGCG     10380

ATCTCGGCCA TGAACTGCTC GATCTCTTCC TCCTGGAGAT CTCCGCGTCC GGCTCGCTCC     10440

ACGGTGGCGG CGAGGTCGTT GGAAATGCGG GCCATGAGCT GCGAGAAGGC GTTGAGGCCT     10500

CCCTCGTTCC AGACGCGGCT GTAGACCACG CCCCCTTCGG CATCGCGGGC GCGCATGACC     10560

ACCTGCGCGA GATTGAGCTC CACGTGCCGG GCGAAGACGG CGTAGTTTCG CAGGCGCTGA     10620

AAGAGGTAGT TGAGGGTGGT GGCGGTGTGT CTGCCACGA AGAAGTACAT AACCCAGCGT      10680

CGCAACGTGG ATTCGTTGAT ATCCCCCAAG GCCTCAAGGC GCTCCATGGC CTCGTAGAAG     10740

TCCACGGCGA AGTTGAAAAA CTGGGAGTTG CGCGCCGACA CGGTTAACTC CTCCTCCAGA     10800

AGACGGATGA GCTCGGCGAC AGTGTCGCGC ACCTCGCGCT CAAAGGCTAC AGGGGCCTCT     10860

TCTTCTTCTT CAATCTCCTC TTCCATAAGG GCCTCCCCTT CTTCTTCTTC TGGCGGCGGT     10920

GGGGGAGGGG GGACACGGCG GCGACGACGG CGCACCGGGA GGCGGTCGAC AAAGCGCTCG     10980

ATCATCTCCC CGCGGCGACG GCGCATGGTC TCGGTGACGG CGCGGCCGTT CTCGCGGGGG     11040

CGCAGTTGGA AGACGCCGCC CGTCATGTCC CGGTTATGGG TTGGCGGGGG GCTGCCATGC     11100

GGCAGGGATA CGGCGCTAAC GATGCATCTC AACAATTGTT GTGTAGGTAC TCCGCCGCCG     11160

AGGGACCTGA GCGAGTCCGC ATCGACCGGA TCGGAAAACC TCTCGAGAAA GGCGTCTAAC     11220

CAGTCACAGT CGCAAGGTAG GCTGAGCACC GTGGCGGGCG GCAGCGGGCG GCGGTCGGGG     11280

TTGTTTCTGG CGGAGGTGCT GCTGATGATG TAATTAAAGT AGGCGGTCTT GAGACGGCGG     11340

ATGGTCGACA GAAGCACCAT GTCCTTGGGT CCGGCCTGCT GAATGCGCAG GCGGTCGGCC     11400

ATGCCCCAGG CTTCGTTTTG ACATCGGCGC AGGTCTTTGT AGTAGTCTTG CATGAGCCTT     11460

TCTACCGGCA CTTCTTCTTC TCCTTCCTCT TGTCCTGCAT CTCTTGCATC TATCGCTGCG     11520

GCGGCGGCGG AGTTTGGCCG TAGGTGGCGC CCTCTTCCTC CCATGCGTGT GACCCCGAAG     11580
```

```
CCCCTCATCG GCTGAAGCAG GGCTAGGTCG GCGACAACGC GCTCGGCTAA TATGGCCTGC   11640
TGCACCTGCG TGAGGGTAGA CTGGAAGTCA TCCATGTCCA CAAAGCGGTG GTATGCGCCC   11700
GTGTTGATGG TGTAAGTGCA GTTGGCCATA ACGGACCAGT TAACGGTCTG GTGACCCGGC   11760
TGCGAGAGCT CGGTGTACCT GAGACGCGAG TAAGCCCTCG AGTCAAATAC GTAGTCGTTG   11820
CAAGTCCGCA CCAGGTACTG GTATCCCACC AAAAAGTGCG GCGGCGGCTG GCGGTAGAGG   11880
GGCCAGCGTA GGGTGGCCGG GGCTCCGGGG GCGAGATCTT CCAACATAAG GCGATGATAT   11940
CCGTAGATGT ACCTGGACAT CCAGGTGATG CCGGCGGCGG TGGTGGAGGC GCGCGGAAAG   12000
TCGCGGACGC GGTTCCAGAT GTTGCGCAGC GGCAAAAAGT GCTCCATGGT CGGGACGCTC   12060
TGGCCGGTCA GGCGCGCGCA ATCGTTGACG CTCTACCGTG CAAAAGGAGA GCCTGTAAGC   12120
GGGCACTCTT CCGTGGTCTG GTGGATAAAT TCGCAAGGGT ATCATGGCGG ACGACCGGGG   12180
TTCGAGCCCC GTATCCGGCC GTCCGCCGTG ATCCATGCGG TTACCGCCCG CGTGTCGAAC   12240
CCAGGTGTGC GACGTCAGAC AACGGGGGAG TGCTCCTTTT GGCTTCCTTC CAGGCGCGGC   12300
GGCTGCTGCG CTAGCTTTTT TGGCCACTGG CCGCGCGCAG CGTAAGCGGT TAGGCTGGAA   12360
AGCGAAAGCA TTAAGTGGCT CGCTCCCTGT AGCCGGAGGG TTATTTTCCA AGGGTTGAGT   12420
CGCGGGACCC CCGGTTCGAG TCTCGGACCG GCCGGACTGC GGCGAACGGG GGTTTGCCTC   12480
CCCGTCATGC AAGACCCCGC TTGCAAATTC CTCCGGAAAC AGGGACGAGC CCCTTTTTTG   12540
CTTTTCCCAG ATGCATCCGG TGCTGCGGCA GATGCGCCCC CCTCCTCAGC AGCGGCAAGA   12600
GCAAGAGCAG CGGCAGACAT GCAGGGCACC CTCCCCTCCT CCTACCGCGT CAGGAGGGGC   12660
GACATCCGCG GTTGACGCGG CAGCAGATGG TGATTACGAA CCCCCGCGGC GCCGGGCCCG   12720
GCACTACCTG GACTTGGAGG AGGGCGAGGG CCTGGCGCGG CTAGGAGCGC CCTCTCCTGA   12780
GCGGTACCCA AGGGTGCAGC TGAAGCGTGA TACGCGTGAG GCGTACGTGC CGCGGCAGAA   12840
CCTGTTTCGC GACCGCGAGG GAGAGGAGCC CGAGGAGATG CGGGATCGAA AGTTCCACGC   12900
AGGGCGCGAG CTGCGGCATG GCCTGAATCG CGAGCGGTTG CTGCGCGAGG AGGACTTTGA   12960
GCCCGACGCG CGAACCGGGA TTAGTCCCGC GCGCGCACAC GTGGCGGCCG CCGACCTGGT   13020
AACCGCATAC GAGCAGACGG TGAACCAGGA GATTAACTTT CAAAAAAGCT TTAACAACCA   13080
CGTGCGTACG CTTGTGGCGC GCGAGGAGGT GGCTATAGGA CTGATGCATC TGTGGGACTT   13140
TGTAAGCGCG CTGGAGCAAA ACCCAAATAG CAAGCCGCTC ATGGCGCAGC TGTTCCTTAT   13200
AGTGCAGCAC AGCAGGGACA ACGAGGCATT CAGGGATGCG CTGCTAAACA TAGTAGAGCC   13260
CGAGGGCCGC TGGCTGCTCG ATTTGATAAA CATCCTGCAG AGCATAGTGG TGCAGGAGCG   13320
CAGCTTGAGC CTGGCTGACA AGGTGGCCGC CATCAACTAT TCCATGCTTA GCCTGGGCAA   13380
GTTTTACGCC CGCAAGATAT ACCATACCCC TTACGTTCCC ATAGACAAGG AGGTAAAGAT   13440
CGAGGGGTTC TACATGCGCA TGGCGCTGAA GGTGCTTACC TTGAGCGACG ACCTGGGCGT   13500
TTATCGCAAC GAGCGCATCC ACAAGGCCGT GAGCGTGAGC CGGCGGCGCG AGCTCAGCGA   13560
CCGCGAGCTG ATGCACAGCC TGCAAAGGGC CCTGGCTGGC ACGGGCAGCG GCGATAGAGA   13620
GGCCGAGTCC TACTTTGACG CGGGCGCTGA CCTGCGCTGG GCCCCAAGCC GACGCGCCCT   13680
```

```
GGAGGCAGCT GGGGCCGGAC CTGGGCTGGC GGTGGCACCC GCGCGCGCTG GCAACGTCGG  13740
CGGCGTGGAG GAATATGACG AGGACGATGA GTACGAGCCA GAGGACGGCG AGTACTAAGC  13800
GGTGATGTTT CTGATCAGAT GATGCAAGAC GCAACGGACC CGGCGGTGCG GGCGGCGCTG  13860
CAGAGCCAGC CGTCCGGCCT TAACTCCACG GACGACTGGC GCCAGGTCAT GGACCGCATC  13920
ATGTCGCTGA CTGCGCGCAA TCCTGACGCG TTCGGCAGC AGCCGCAGGC CAACCGGCTC  13980
TCCGCAATTC TGGAAGCGGT GGTCCCGGCG CGCGCAAACC CCACGCACGA GAAGGTGCTG  14040
GCGATCGTAA ACGCGCTGGC CGAAAACAGG GCCATCCGGC CCGACGAGGC CGGCCTGGTC  14100
TACGACGCGC TGCTTCAGCG CGTGGCTCGT TACAACAGCG GCAACGTGCA GACCAACCTG  14160
GACCGGCTGG TGGGGGATGT GCGCGAGGCC GTGGCGCAGC GTGAGCGCGC GCAGCAGCAG  14220
GGCAACCTGG GCTCCATGGT TGCACTAAAC GCCTTCCTGA GTACACAGCC CGCCAACGTG  14280
CCGCGGGGAC AGGAGGACTA CACCAACTTT GTGAGCGCAC TGCGGCTAAT GGTGACTGAG  14340
ACACCGCAAA GTGAGGTGTA CCAGTCTGGG CCAGACTATT TTTTCCAGAC CAGTAGACAA  14400
GGCCTGCAGA CCGTAAACCT GAGCCAGGCT TTCAAAAACT TGCAGGGGCT GTGGGGGGTG  14460
CGGGCTCCCA CAGGCGACCG CGCGACCGTG TCTAGCTTGC TGACGCCCAA CTCGCGCCTG  14520
TTGCTGCTGC TAATAGCGCC CTTCACGGAC AGTGGCAGCG TGTCCCGGGA CACATACCTA  14580
GGTCACTTGC TGACACTGTA CCGCGAGGCC ATAGGTCAGG CGCATGTGGA CGAGCATACT  14640
TTCCAGGAGA TTACAAGTGT CAGCCGCGCG CTGGGGCAGG AGGACACGGG CAGCCTGGAG  14700
GCAACCCTAA ACTACCTGCT GACCAACCGG CGGCAGAAGA TCCCCTCGTT GCACAGTTTA  14760
AACAGCGAGG AGGAGCGCAT TTTGCGCTAC GTGCAGCAGA GCGTGAGCCT TAACCTGATG  14820
CGCGACGGGG TAACGCCCAG CGTGGCGCTG GACATGACCG CGCGCAACAT GGAACCGGGC  14880
ATGTATGCCT CAAACCGGCC GTTTATCAAC CGCCTAATGG ACTACTTGCA TCGCGCGGCC  14940
GCCGTGAACC CCGAGTATTT CACCAATGCC ATCTTGAACC CGCACTGGCT ACCGCCCCCT  15000
GGTTTCTACA CCGGGGGATT CGAGGTGCCC GAGGGTAACG ATGGATTCCT CTGGGACGAC  15060
ATAGACGACA GCGTGTTTTC CCCGCAACCG CAGACCCTGC TAGAGTTGCA ACAGCGCGAG  15120
CAGGCAGAGG CGGCGCTGCG AAAGGAAAGC TTCCGCAGGC CAAGCAGCTT GTCCGATCTA  15180
GGCGCTGCGG CCCCGCGGTC AGATGCTAGT AGCCCATTTC CAAGCTTGAT AGGGTCTCTT  15240
ACCAGCACTC GCACCACCCG CCCGCGCCTG CTGGGCGAGG AGGAGTACCT AAACAACTCG  15300
CTGCTGCAGC CGCAGCGCGA AAAAAACCTG CCTCCGGCAT TTCCCAACAA CGGGATAGAG  15360
AGCCTAGTGG ACAAGATGAG TAGATGGAAG ACGTACGCGC AGGAGCACAG GGACGTGCCA  15420
GGCCCGCGCC CGCCCACCCG TCGTCAAAGG CACGACCGTC AGCGGGGTCT GGTGTGGGAG  15480
GACGATGACT CGGCAGACGA CAGCAGCGTC CTGGATTTGG GAGGGAGTGG CAACCCGTTT  15540
GCGCACCTTC GCCCCAGGCT GGGGAGAATG TTTTAAAAAA AAAAAAGCAT GATGCAAAAT  15600
AAAAAACTCA CCAAGGCCAT GGCACCGAGC GTTGGTTTTC TTGTATTCCC CTTAGTATGC  15660
GGCGCGCGGC GATGTATGAG GAAGGTCCTC CTCCCTCCTA CGAGAGTGTG GTGAGCGCGG  15720
CGCCAGTGGC GGCGGCGCTG GGTTCTCCCT TCGATGCTCC CCTGGACCCG CCGTTTGTGC  15780
```

```
CTCCGCGGTA CCTGCGGCCT ACCGGGGGGA GAAACAGCAT CCGTTACTCT GAGTTGGCAC   15840
CCCTATTCGA CACCACCCGT GTGTACCTGG TGGACAACAA GTCAACGGAT GTGGCATCCC   15900
TGAACTACCA GAACGACCAC AGCAACTTTC TGACCACGGT CATTCAAAAC AATGACTACA   15960
GCCCGGGGGA GGCAAGCACA CAGACCATCA ATCTTGACGA CCGGTCGCAC TGGGGCGGCG   16020
ACCTGAAAAC CATCCTGCAT ACCAACATGC CAAATGTGAA CGAGTTCATG TTTACCAATA   16080
AGTTTAAGGC GCGGGTGATG GTGTCGCGCT TGCCTACTAA GGACAATCAG GTGGAGCTGA   16140
AATACGAGTG GGTGGAGTTC ACGCTGCCCG AGGGCAACTA CTCCGAGACC ATGACCATAG   16200
ACCTTATGAA CAACGCGATC GTGGAGCACT ACTTGAAAGT GGGCAGACAG AACGGGGTTC   16260
TGGAAAGCGA CATCGGGGTA AAGTTTGACA CCCGCAACTT CAGACTGGGG TTTGACCCCG   16320
TCACTGGTCT TGTCATGCCT GGGGTATATA CAAACGAAGC CTTCCATCCA GACATCATTT   16380
TGCTGCCAGG ATGCGGGGTG GACTTCACCC ACAGCCGCCT GAGCAACTTG TTGGGCATCC   16440
GCAAGCGGCA ACCCTTCCAG GAGGGCTTTA GGATCACCTA CGATGATCTG GAGGGTGGTA   16500
ACATTCCCGC ACTGTTGGAT GTGGACGCCT ACCAGGCGAG CTTGAAAGAT GACACCGAAC   16560
AGGGCGGGGG TGGCGCAGGC GGCAGCAACA GCAGTGGCAG CGGCGCGGAA GAGAACTCCA   16620
ACGCGGCAGC CGCGGCAATG CAGCCGGTGG AGGACATGAA CGATCATGCC ATTCGCGGCG   16680
ACACCTTTGC CACACGGGCT GAGGAGAAGC GCGCTGAGGC CGAAGCAGCG GCCGAAGCTG   16740
CCGCCCCCGC TGCGCAACCC GAGGTCGAGA AGCCTCAGAA GAAACCGGTG ATCAAACCCC   16800
TGACAGAGGA CAGCAAGAAA CGCAGTTACA ACCTAATAAG CAATGACAGC ACCTTCACCC   16860
AGTACCGCAG CTGGTACCTT GCATACAACT ACGGCGACCC TCAGACCGGA ATCCGCTCAT   16920
GGACCCTGCT TTGCACTCCT GACGTAACCT GCGGCTCGGA GCAGGTCTAC TGGTCGTTGC   16980
CAGACATGAT GCAAGACCCC GTGACCTTCC GCTCCACGCG CCAGATCAGC AACTTTCCGG   17040
TGGTGGGCGC CGAGCTGTTG CCCGTGCACT CCAAGAGCTT CTACAACGAC CAGGCCGTCT   17100
ACTCCCAACT CATCCGCCAG TTTACCTCTC TGACCCACGT GTTCAATCGC TTTCCCGAGA   17160
ACCAGATTTT GGCGCGCCCG CCAGCCCCCA CCATCACCAC CGTCAGTGAA AACGTTCCTG   17220
CTCTCACAGA TCACGGGACG CTACCGCTGC GCAACAGCAT CGGAGGAGTC CAGCGAGTGA   17280
CCATTACTGA CGCCAGACGC CGCACCTGCC CCTACGTTTA CAAGGCCCTG GGCATAGTCT   17340
CGCCGCGCGT CCTATCGAGC CGCACTTTTT GAGCAAGCAT GTCCATCCTT ATATCGCCCA   17400
GCAATAACAC AGGCTGGGGC CTGCGCTTCC CAAGCAAGAT GTTTGGCGGG GCCAAGAAGC   17460
GCTCCGACCA ACACCCAGTG CGCGTGCGCG GCACTACCG CGCGCCCTGG GGCGCGCACA    17520
AACGCGGCCG CACTGGGCGC ACCACCGTCG ATGACGCCAT CGACGCGGTG GTGGAGGAGG   17580
CGCGCAACTA CACGCCCACG CCGCCACCAG TGTCCACAGT GGACGCGGCC ATTCAGACCG   17640
TGGTGCGCGG AGCCCGGCGC TATGCTAAAA TGAAGAGACG GCGGAGGCGC GTAGCACGTC   17700
GCCACCGCCG CCGACCCGGC ACTGCCGCCC AACGCGCGGC GGCGGCCCTG CTTAACCGCG   17760
CACGTCGCAC CGGCCGACGG GCGGCCATGC GGGCCGCTCG AAGGCTGGCC GCGGGTATTG   17820
TCACTGTGCC CCCCAGGTCC AGGCGACGAG CGGCCGCCGC AGCAGCCGCG GCCATTAGTG   17880
```

- 95 -

```
CTATGACTCA GGGTCGCAGG GGCAACGTGT ATTGGGTGCG CGACTCGGTT AGCGGCCTGC    17940

GCGTGCCCGT GCGCACCCGC CCCCCGCGCA ACTAGATTGC AAGAAAAAAC TACTTAGACT    18000

CGTACTGTTG TATGTATCCA GCGGCGGCGG CGCGCAACGA AGCTATGTCC AAGCGCAAAA    18060

TCAAAGAAGA GATGCTCCAG GTCATCGCGC CGGAGATCTA TGGCCCCCCG AAGAAGGAAG    18120

AGCAGGATTA CAAGCCCGA AAGCTAAAGC GGGTCAAAAA GAAAAGAAA GATGATGATG      18180

ATGAACTTGA CGACGAGGTG GAACTGCTGC ACGCTACCGC GCCCAGGCGA CGGGTACAGT    18240

GGAAAGGTCG ACGCGTAAAA CGTGTTTTGC GACCCGGCAC CACCGTAGTC TTTACGCCCG    18300

GTGAGCGCTC CACCCGCACC TACAAGCGCG TGTATGATGA GGTGTACGGC GACGAGGACC    18360

TGCTTGAGCA GGCCAACGAG CGCCTCGGGG AGTTTGCCTA CGGAAAGCGG CATAAGGACA    18420

TGCTGGCGTT GCCGCTGGAC GAGGGCAACC CAACACCTAG CCTAAAGCCC GTAACACTGC    18480

AGCAGGTGCT GCCCGCGCTT GCACCGTCCG AAGAAAAGCG CGGCCTAAAG CGCGAGTCTG    18540

GTGACTTGGC ACCCACCGTG CAGCTGATGG TACCCAAGCG CCAGCGACTG GAAGATGTCT    18600

TGGAAAAAAT GACCGTGGAA CCTGGGCTGG AGCCCGAGGT CCGCGTGCGG CCAATCAAGC    18660

AGGTGGCGCC GGGACTGGGC GTGCAGACCG TGGACGTTCA GATACCCACT ACCAGTAGCA    18720

CCAGTATTGC CACCGCCACA GAGGGCATGG AGACACAAAC GTCCCCGGTT GCCTCAGCGG    18780

TGGCGGATGC CGCGGTGCAG GCGGTCGCTG CGGCCGCGTC CAAGACCTCT ACGGAGGTGC    18840

AAACGGACCC GTGGATGTTT CGCGTTTCAG CCCCCCGGCG CCCGCGCGGT TCGAGGAAGT    18900

ACGGCGCCGC CAGCGCGCTA CTGCCCGAAT ATGCCCTACA TCCTTCCATT GCGCCTACCC    18960

CCGGCTATCG TGGCTACACC TACCGCCCCA GAAGACGAGC AACTACCCGA CGCCGAACCA    19020

CCACTGGAAC CCGCCGCCGC CGTCGCCGTC GCCAGCCCGT GCTGGCCCCG ATTTCCGTGC    19080

GCAGGGTGGC TCGCGAAGGA GGCAGGACCC TGGTGCTGCC AACAGCGCGC TACCACCCCA    19140

GCATCGTTTA AAAGCCGGTC TTTGTGGTTC TTGCAGATAT GGCCCTCACC TGCCGCCTCC    19200

GTTTCCCGGT GCCGGGATTC CGAGGAAGAA TGCACCGTAG GAGGGGCATG GCCGGCCACG    19260

GCCTGACGGG CGGCATGCGT CGTGCGCACC ACCGGCGGCG GCGCGCGTCG CACCGTCGCA    19320

TGCGCGGCGG TATCCTGCCC CTCCTTATTC CACTGATCGC CGCGGCGATT GGCGCCGTGC    19380

CCGGAATTGC ATCCGTGGCC TTGCAGGCGC AGAGACACTG ATTAAAAACA AGTTGCATGT    19440

GGAAAAATCA AAATAAAAAG TCTGGACTCT CACGCTCGCT TGGTCCTGTA ACTATTTTGT    19500

AGAATGGAAG ACATCAACTT TGCGTCTCTG GCCCCGCGAC ACGGCTCGCG CCCGTTCATG    19560

GGAAACTGGC AAGATATCGG CACCAGCAAT ATGAGCGGTG GCGCCTTCAG CTGGGGCTCG    19620

CTGTGGAGCG GCATTAAAAA TTTCGGTTCC ACCGTTAAGA ACTATGGCAG CAAGGCCTGG    19680

AACAGCAGCA CAGGCCAGAT GCTGAGGGAT AAGTTGAAAG AGCAAAATTT CCAACAAAAG    19740

GTGGTAGATG GCCTGGCCTC TGGCATTAGC GGGGTGGTGG ACCTGGCCAA CCAGGCAGTG    19800

CAAAATAAGA TTAACAGTAA GCTTGATCCC CGCCCTCCCG TAGAGGAGCC TCCACCGGCC    19860

GTGGAGACAG TGTCTCCAGA GGGGCGTGGC GAAAAGCGTC CGCGCCCCGA CAGGGAAGAA    19920

ACTCTGGTGA CGCAAATAGA CGAGCCTCCC TCGTACGAGG AGGCACTAAA GCAAGGCCTG    19980
```

```
CCCACCACCC GTCCCATCGC GCCCATGGCT ACCGGAGTGC TGGGCCAGCA CACACCCGTA    20040
ACGCTGGACC TGCCTCCCCC CGCCGACACC CAGCAGAAAC CTGTGCTGCC AGGCCCGACC    20100
GCCGTTGTTG TAACCCGTCC TAGCCGCGCG TCCCTGCGCC GCGCCGCCAG CGGTCCGCGA    20160
TCGTTGCGGC CCGTAGCCAG TGGCAACTGG CAAAGCACAC TGAACAGCAT CGTGGGTCTG    20220
GGGGTGCAAT CCCTGAAGCG CCGACGATGC TTCTGAATAG CTAACGTGTC GTATGTGTGT    20280
CATGTATGCG TCCATGTCGC CGCCAGAGGA GCTGCTGAGC CGCCGCGCGC CGCTTTCCA     20340
AGATGGCTAC CCCTTCGATG ATGCCGCAGT GGTCTTACAT GCACATCTCG GCCAGGACG     20400
CCTCGGAGTA CCTGAGCCCC GGGCTGGTGC AGTTTGCCCG CGCCACCGAG ACGTACTTCA    20460
GCCTGAATAA CAAGTTTAGA AACCCCACGG TGGCGCCTAC GCACGACGTG ACCACAGACC    20520
GGTCCCAGCG TTTGACGCTG CGGTTCATCC CTGTGGACCG TGAGGATACT GCGTACTCGT    20580
ACAAGGCGCG GTTCACCCTA GCTGTGGGTG ATAACCGTGT GCTGGACATG GCTTCCACGT    20640
ACTTTGACAT CCGCGGCGTG CTGGACAGGG GCCCTACTTT TAAGCCCTAC TCTGGCACTG    20700
CCTACAACGC CCTGGCTCCC AAGGGTGCCC CAAATCCTTG CGAATGGGAT GAAGCTGCTA    20760
CTGCTCTTGA AATAAACCTA GAAGAAGAGG ACGATGACAA CGAAGACGAA GTAGACGAGC    20820
AAGCTGAGCA GCAAAAAACT CACGTATTTG GGCAGGCGCC TTATTCTGGT ATAAATATTA    20880
CAAAGGAGGG TATTCAAATA GGTGTCGAAG GTCAAACACC TAAATATGCC GATAAAACAT    20940
TTCAACCTGA ACCTCAAATA GGAGAATCTC AGTGGTACGA AACTGAAATT AATCATGCAG    21000
CTGGGAGAGT CCTTAAAAAG ACTACCCCAA TGAAACCATG TTACGGTTCA TATGCAAAAC    21060
CCACAAATGA AAATGGAGGG CAAGGCATTC TTGTAAAGCA ACAAAATGGA AAGCTAGAAA    21120
GTCAAGTGGA AATGCAATTT TTCTCAACTA CTGAGGCGAC CGCAGGCAAT GGTGATAACT    21180
TGACTCCTAA AGTGGTATTG TACAGTGAAG ATGTAGATAT AGAAACCCCA GACACTCATA    21240
TTTCTTACAT GCCCACTATT AAGGAAGGTA ACTCACGAGA ACTAATGGGC CAACAATCTA    21300
TGCCCAACAG GCCTAATTAC ATTGCTTTTA GGGACAATTT TATTGGTCTA ATGTATTACA    21360
ACAGCACGGG TAATATGGGT GTTCTGGCGG GCCAAGCATC GCAGTTGAAT GCTGTTGTAG    21420
ATTTGCAAGA CAGAAACACA GAGCTTTCAT ACCAGCTTTT GCTTGATTCC ATTGGTGATA    21480
GAACCAGGTA CTTTTCTATG TGGAATCAGG CTGTTGACAG CTATGATCCA GATGTTAGAA    21540
TTATTGAAAA TCATGGAACT GAAGATGAAC TTCCAAATTA CTGCTTTCCA CTGGGAGGTG    21600
TGATTAATAC AGAGACTCTT ACCAAGGTAA AACCTAAAAC AGGTCAGGAA AATGGATGGG    21660
AAAAAGATGC TACAGAATTT TCAGATAAAA ATGAAATAAG AGTTGGAAAT AATTTTGCCA    21720
TGGAAATCAA TCTAAATGCC AACCTGTGGA GAAATTTCCT GTACTCCAAC ATAGCGCTGT    21780
ATTTGCCCGA CAAGCTAAAG TACAGTCCTT CCAACGTAAA AATTTCTGAT AACCCAAACA    21840
CCTACGACTA CATGAACAAG CGAGTGGTGG CTCCCGGGTT AGTGGACTGC TACATTAACC    21900
TTGGAGCACG CTGGTCCCTT GACTATATGG ACAACGTCAA CCCATTTAAC CACCACCGCA    21960
ATGCTGGCCT GCGCTACCGC TCAATGTTGC TGGGCAATGG TCGCTATGTG CCCTTCCACA    22020
TCCAGGTGCC TCAGAAGTTC TTTGCCATTA AAAACCTCCT TCTCCTGCCG GGCTCATACA    22080
```

●   ●

```
CCTACGAGTG GAACTTCAGG AAGGATGTTA ACATGGTTCT GCAGAGCTCC CTAGGAAATG    22140

ACCTAAGGGT TGACGGAGCC AGCATTAAGT TTGATAGCAT TTGCCTTTAC GCCACCTTCT    22200

TCCCCATGGC CCACAACACC GCCTCCACGC TTGAGGCCAT GCTTAGAAAC GACACCAACG    22260

ACCAGTCCTT TAACGACTAT CTCTCCGCCG CCAACATGCT CTACCCTATA CCCGCCAACG    22320

CTACCAACGT GCCCATATCC ATCCCTCCC GCAACTGGGC GGCTTTCCGC GGCTGGGCCT    22380

TCACGCGCCT TAAGACTAAG GAAACCCCAT CACTGGGCTC GGGCTACGAC CCTTATTACA    22440

CCTACTCTGG CTCTATACCC TACCTAGATG GAACCTTTTA CCTCAACCAC ACCTTTAAGA    22500

AGGTGGCCAT TACCTTTGAC TCTTCTGTCA GCTGGCCTGG CAATGACCGC CTGCTTACCC    22560

CCAACGAGTT TGAAATTAAG CGCTCAGTTG ACGGGGAGGG TTACAACGTT GCCCAGTGTA    22620

ACATGACCAA AGACTGGTTC CTGGTACAAA TGCTAGCTAA CTACAACATT GGCTACCAGG    22680

GCTTCTATAT CCCAGAGAGC TACAAGGACC GCATGTACTC CTTCTTTAGA AACTTCCAGC    22740

CCATGAGCCG TCAGGTGGTG GATGATACTA AATACAAGGA CTACCAACAG GTGGGCATCC    22800

TACACCAACA CAACAACTCT GGATTTGTTG GCTACCTTGC CCCCACCATG CGCGAAGGAC    22860

AGGCCTACCC TGCTAACTTC CCCTATCCGC TTATAGGCAA GACCGCAGTT GACAGCATTA    22920

CCCAGAAAAA GTTTCTTTGC GATCGCACCC TTTGGCGCAT CCCATTCTCC AGTAACTTTA    22980

TGTCCATGGG CGCACTCACA GACCTGGGCC AAAACCTTCT CTACGCCAAC TCCGCCCACG    23040

CGCTAGACAT GACTTTTGAG GTGGATCCCA TGGACGAGCC CACCCTTCTT TATGTTTTGT    23100

TTGAAGTCTT TGACGTGGTC CGTGTGCACC GGCCGCACCG CGGCGTCATC GAAACCGTGT    23160

ACCTGCGCAC GCCCTTCTCG GCCGGCAACG CCACAACATA AAGAAGCAAG CAACATCAAC    23220

AACAGCTGCC GCCATGGGCT CCAGTGAGCA GGAACTGAAA GCCATTGTCA AAGATCTTGG    23280

TTGTGGGCCA TATTTTTTGG GCACCTATGA CAAGCGCTTT CCAGGCTTTG TTTCTCCACA    23340

CAAGCTCGCC TGCGCCATAG TCAATACGGC CGGTCGCGAG ACTGGGGGCG TACACTGGAT    23400

GGCCTTTGCC TGGAACCCGC ACTCAAAAAC ATGCTACCTC TTTGAGCCCT TTGGCTTTTC    23460

TGACCAGCGA CTCAAGCAGG TTTACCAGTT TGAGTACGAG TCACTCCTGC GCCGTAGCGC    23520

CATTGCTTCT TCCCCCGACC GCTGTATAAC GCTGGAAAAG TCCACCCAAA GCGTACAGGG    23580

GCCCAACTCG GCCGCCTGTG GACTATTCTG CTGCATGTTT CTCCACGCCT TTGCCAACTG    23640

GCCCCAAACT CCCATGGATC ACAACCCCAC CATGAACCTT ATTACCGGGG TACCCAACTC    23700

CATGCTCAAC AGTCCCCAGG TACAGCCCAC CCTGCGTCGC AACCAGGAAC AGCTCTACAG    23760

CTTCCTGGAG CGCCACTCGC CCTACTTCCG CAGCCACAGT GCGCAGATTA GGAGCGCCAC    23820

TTCTTTTTGT CACTTGAAAA ACATGTAAAA ATAATGTACT AGAGACACTT TCAATAAAGG    23880

CAAATGCTTT TATTTGTACA CTCTCGGGTG ATTATTTACC CCCACCCTTG CCGTCTGCGC    23940

CGTTTAAAAA TCAAAGGGGT TCTGCCGCGC ATCGCTATGC GCCACTGGCA GGGACACGTT    24000

GCGATACTGG TGTTTAGTGC TCCACTTAAA CTCAGGCACA ACCATCCGCG GCAGCTCGGT    24060

GAAGTTTTCA CTCCACAGGC TGCGCACCAT CACCAACGCG TTTAGCAGGT CGGGCGCCGA    24120

TATCTTGAAG TCGCAGTTGG GGCCTCCGCC CTGCGCGCGC GAGTTGCGAT ACACAGGGTT    24180
```

```
GCAGCACTGG AACACTATCA GCGCCGGGTG GTGCACGCTG GCCAGCACGC TCTTGTCGGA   24240

GATCAGATCC GCGTCCAGGT CCTCCGCGTT GCTCAGGGCG AACGGAGTCA ACTTTGGTAG   24300

CTGCCTTCCC AAAAAGGGCG CGTGCCCAGG CTTTGAGTTG CACTCGCACC GTAGTGGCAT   24360

CAAAAGGTGA CCGTGCCCGG TCTGGGCGTT AGGATACAGC GCCTGCATAA AAGCCTTGAT   24420

CTGCTTAAAA GCCACCTGAG CCTTTGCGCC TTCAGAGAAG AACATGCCGC AAGACTTGCC   24480

GGAAAACTGA TTGGCCGGAC AGGCCGCGTC GTGCACGCAG CACCTTGCGT CGGTGTTGGA   24540

GATCTGCACC ACATTTCGGC CCCACCGGTT CTTCACGATC TTGGCCTTGC TAGACTGCTC   24600

CTTCAGCGCG CGCTGCCCGT TTTCGCTCGT CACATCCATT TCAATCACGT GCTCCTTATT   24660

TATCATAATG CTTCCGTGTA GACACTTAAG CTCGCCTTCG ATCTCAGCGC AGCGGTGCAG   24720

CCACAACGCG CAGCCCGTGG GCTCGTGATG CTTGTAGGTC ACCTCTGCAA ACGACTGCAG   24780

GTACGCCTGC AGGAATCGCC CCATCATCGT CACAAAGGTC TTGTTGCTGG TGAAGGTCAG   24840

CTGCAACCCG CGGTGCTCCT CGTTCAGCCA GGTCTTGCAT ACGGCCGCCA GAGCTTCCAC   24900

TTGGTCAGGC AGTAGTTTGA AGTTCGCCTT TAGATCGTTA TCCACGTGGT ACTTGTCCAT   24960

CAGCGCGCGC GCAGCCTCCA TGCCCTTCTC CCACGCAGAC ACGATCGGCA CACTCAGCGG   25020

GTTCATCACC GTAATTTCAC TTTCCGCTTC GCTGGGCTCT TCCTCTTCCT CTTGCGTCCG   25080

CATACCACGC GCCACTGGGT CGTCTTCATT CAGCCGCCGC ACTGTGCGCT TACCTCCTTT   25140

GCCATGCTTG ATTAGCACCG GTGGGTTGCT GAAACCCACC ATTTGTAGCG CCACATCTTC   25200

TCTTTCTTCC TCGCTGTCCA CGATTACCTC TGGTGATGGC GGGCGCTCGG GCTTGGGAGA   25260

AGGGCGCTTC TTTTTCTTCT TGGGCGCAAT GGCCAAATCC GCCGCCGAGG TCGATGGCCG   25320

CGGGCTGGGT GTGCGCGGCA CCAGCGCGTC TTGTGATGAG TCTTCCTCGT CCTCGGACTC   25380

GATACGCCGC CTCATCCGCT TTTTTGGGGG CGCCCGGGGA GGCGGCGGCG ACGGGGACGG   25440

GGACGACACG TCCTCCATGG TTGGGGGACG TCGCGCCGCA CCGCGTCCGC GCTCGGGGGT   25500

GGTTTCGCGC TGCTCCTCTT CCCGACTGGC CATTTCCTTC TCCTATAGGC AGAAAAAGAT   25560

CATGGAGTCA GTCGAGAAGA AGGACAGCCT AACCGCCCCC TCTGAGTTCG CCACCACCGC   25620

CTCCACCGAT GCCGCCAACG CGCCTACCAC CTTCCCCGTC GAGGCACCCC CGCTTGAGGA   25680

GGAGGAAGTG ATTATCGAGC AGGACCCAGG TTTTGTAAGC GAAGACGACG AGGACCGCTC   25740

AGTACCAACA GAGGATAAAA AGCAAGACCA GGACAACGCA GAGGCAAACG AGGAACAAGT   25800

CGGGCGGGGG GACGAAAGGC ATGGCGACTA CCTAGATGTG GGAGACGACG TGCTGTTGAA   25860

GCATCTGCAG CGCCAGTGCG CCATTATCTG CGACGCGTTG CAAGAGCGCA GCGATGTGCC   25920

CCTCGCCATA GCGGATGTCA GCCTTGCCTA CGAACGCCAC CTATTCTCAC CGCGCGTACC   25980

CCCCAAACGC CAAGAAAACG GCACATGCGA GCCCAACCCG CGCCTCAACT TCTACCCCGT   26040

ATTTGCCGTG CCAGAGGTGC TTGCCACCTA TCACATCTTT TTCCAAAACT GCAAGATACC   26100

CCTATCCTGC CGTGCCAACC GCAGCCGAGC GGACAAGCAG CTGGCCTTGC GGCAGGGCGC   26160

TGTCATACCT GATATCGCCT CGCTCAACGA AGTGCCAAAA ATCTTTGAGG GTCTTGGACG   26220

CGACGAGAAG CGCGCGGCAA ACGCTCTGCA ACAGGAAAAC AGCGAAAATG AAAGTCACTC   26280
```

```
TGGAGTGTTG GTGGAACTCG AGGGTGACAA CGCGCGCCTA GCCGTACTAA AACGCAGCAT    26340

CGAGGTCACC CACTTTGCCT ACCCGGCACT TAACCTACCC CCCAAGGTCA TGAGCACAGT    26400

CATGAGTGAG CTGATCGTGC GCCGTGCGCA GCCCCTGGAG AGGGATGCAA ATTTGCAAGA    26460

ACAAACAGAG GAGGGCCTAC CCGCAGTTGG CGACGAGCAG CTAGCGCGCT GGCTTCAAAC    26520

GCGCGAGCCT GCCGACTTGG AGGAGCGACG CAAACTAATG ATGGCCGCAG TGCTCGTTAC    26580

CGTGGAGCTT GAGTGCATGC AGCGGTTCTT TGCTGACCCG GAGATGCAGC GCAAGCTAGA    26640

GGAAACATTG CACTACACCT TTCGACAGGG CTACGTACGC CAGGCCTGCA AGATCTCCAA    26700

CGTGGAGCTC TGCAACCTGG TCTCCTACCT TGGAATTTTG CACGAAAACC GCCTTGGGCA    26760

AAACGTGCTT CATTCCACGC TCAAGGGCGA GGCGCGCCGC GACTACGTCC GCGACTGCGT    26820

TTACTTATTT CTATGCTACA CCTGGCAGAC GGCCATGGGC GTTTGGCAGC AGTGCTTGGA    26880

GGAGTGCAAC CTCAAGGAGC TGCAGAAACT GCTAAAGCAA AACTTGAAGG ACCTATGGAC    26940

GGCCTTCAAC GAGCGCTCCG TGGCCGCGCA CCTGGCGGAC ATCATTTTCC CCGAACGCCT    27000

GCTTAAAACC CTGCAACAGG GTCTGCCAGA CTTCACCAGT CAAAGCATGT TGCAGAACTT    27060

TAGGAACTTT ATCCTAGAGC GCTCAGGAAT CTTGCCCGCC ACCTGCTGTG CACTTCCTAG    27120

CGACTTTGTG CCCATTAAGT ACCGCGAATG CCCTCCGCCG CTTTGGGGCC ACTGCTACCT    27180

TCTGCAGCTA GCCAACTACC TTGCCTACCA CTCTGACATA ATGGAAGACG TGAGCGGTGA    27240

CGGTCTACTG GAGTGTCACT GTCGCTGCAA CCTATGCACC CCGCACCGCT CCCTGGTTTG    27300

CAATTCGCAG CTGCTTAACG AAAGTCAAAT TATCGGTACC TTTGAGCTGC AGGGTCCCTC    27360

GCCTGACGAA AAGTCCGCGG CTCCGGGGTT GAAACTCACT CCGGGGCTGT GGACGTCGGC    27420

TTACCTTCGC AAATTTGTAC CTGAGGACTA CCACGCCCAC GAGATTAGGT TCTACGAAGA    27480

CCAATCCCGC CCGCCAAATG CGGAGCTTAC CGCCTGCGTC ATTACCCAGG GCCACATTCT    27540

TGGCCAATTG CAAGCCATCA ACAAAGCCCG CCAAGAGTTT CTGCTACGAA AGGGACGGGG    27600

GGTTTACTTG GACCCCCAGT CCGGCGAGGA GCTCAACCCA ATCCCCCGC CGCCGCAGCC    27660

CTATCAGCAG CAGCCGCGGG CCCTTGCTTC CCAGGATGGC ACCCAAAAAG AAGCTGCAGC    27720

TGCCGCCGCC ACCCACGGAC GAGGAGGAAT ACTGGGACAG TCAGGCAGAG GAGGTTTTGG    27780

ACGAGGAGGA GGAGGACATG ATGGAAGACT GGGAGAGCCT AGACGAGGAA GCTTCCGAGG    27840

TCGAAGAGGT GTCAGACGAA ACACCGTCAC CCTCGGTCGC ATTCCCCTCG CCGGCGCCCC    27900

AGAAATCGGC AACCGGTTCC AGCATGGCTA CAACCTCCGC TCCTCAGGCG CCGCCGGCAC    27960

TGCCCGTTCG CCGACCCAAC CGTAGATGGG ACACCACTGG AACCAGGGCC GGTAAGTCCA    28020

AGCAGCCGCC GCCGTTAGCC CAAGAGCAAC AACAGCGCCA AGGCTACCGC TCATGGCGCG    28080

GGCACAAGAA CGCCATAGTT GCTTGCTTGC AAGACTGTGG GGGCAACATC TCCTTCGCCC    28140

GCCGCTTTCT TCTCTACCAT CACGGCGTGG CCTTCCCCCG TAACATCCTG CATTACTACC    28200

GTCATCTCTA CAGCCCATAC TGCACCGGCG GCAGCGGCAG CGGCAGCAAC AGCAGCGGCC    28260

ACACAGAAGC AAAGGCGACC GGATAGCAAG ACTCTGACAA AGCCCAAGAA ATCCACAGCG    28320

GCGGCAGCAG CAGGAGGAGG AGCGCTGCGT CTGGCGCCCA ACGAACCCGT ATCGACCCGC    28380
```

```
GAGCTTAGAA ACAGGATTTT TCCCACTCTG TATGCTATAT TTCAACAGAG CAGGGGCCAA    28440
GAACAAGAGC TGAAAATAAA AAACAGGTCT CTGCGATCCC TCACCCGCAG CTGCCTGTAT    28500
CACAAAAGCG AAGATCAGCT TCGGCGCACG CTGGAAGACG CGGAGGCTCT CTTCAGTAAA    28560
TACTGCGCGC TGACTCTTAA GGACTAGTTT CGCGCCCTTT CTCAAATTTA AGCGCGAAAA    28620
CTACGTCATC TCCAGCGGCC ACACCCGGCG CCAGCACCTG TCGTCAGCGC CATTATGAGC    28680
AAGGAAATTC CCACGCCCTA CATGTGGAGT TACCAGCCAC AAATGGGACT TGCGGCTGGA    28740
GCTGCCCAAG ACTACTCAAC CCGAATAAAC TACATGAGCG CGGGACCCCA CATGATATCC    28800
CGGGTCAACG GAATCCGCGC CCACCGAAAC CGAATTCTCT TGGAACAGGC GGCTATTACC    28860
ACCACACCTC GTAATAACCT TAATCCCCGT AGTTGGCCCG CTGCCCTGGT GTACCAGGAA    28920
AGTCCCGCTC CCACCACTGT GGTACTTCCC AGAGACGCCC AGGCCGAAGT TCAGATGACT    28980
AACTCAGGGG CGCAGCTTGC GGGCGGCTTT CGTCACAGGG TGCGGTCGCC CGGGCAGGGT    29040
ATAACTCACC TGACAATCAG AGGGCGAGGT ATTCAGCTCA ACGACGAGTC GGTGAGCTCC    29100
TCGCTTGGTC TCCGTCCGGA CGGGACATTT CAGATCGGCG GCGCCGGCCG TCCTTCATTC    29160
ACGCCTCGTC AGGCAATCCT AACTCTGCAG ACCTCGTCCT CTGAGCCGCG CTCTGGAGGC    29220
ATTGGAACTC TGCAATTTAT TGAGGAGTTT GTGCCATCGG TCTACTTTAA CCCCTTCTCG    29280
GGACCTCCCG GCCACTATCC GGATCAATTT ATTCCTAACT TTGACGCGGT AAAGGACTCG    29340
GCGGACGGCT ACGACTGAAT GTTAATTAAG TTCCTGTCCA TCCGCACCCA CTATCTTCAT    29400
GTTGTTGCAG ATGAAGCGCG CAAGACCGTC TGAAGATACC TTCAACCCCG TGTATCCATA    29460
TGACACGGAA ACCGGTCCTC CAACTGTGCC TTTTCTTACT CCTCCCTTTG TATCCCCCAA    29520
TGGGTTTCAA GAGAGTCCCC CTGGGGTACT CTCTTTGCGC CTATCCGAAC CTCTAGTTAC    29580
CTCCAATGGC ATGCTTGCGC TCAAAATGGG CAACGGCCTC TCTCTGGACG AGGCCGGCAA    29640
CCTTACCTCC CAAAATGTAA CCACTGTGAG CCCACCTCTC AAAAAAACCA AGTCAAACAT    29700
AAACCTGGAA ATATCTGCAC CCCTCACAGT TACCTCAGAA GCCCTAACTG TGGCTGCCGC    29760
CGCACCTCTA ATGGTCGCGG GCAACACACT CACCATGCAA TCACAGGCCC CGCTAACCGT    29820
GCACGACTCC AAACTTAGCA TTGCCACCCA AGGACCCCTC ACAGTGTCAG AAGGAAAGCT    29880
AGCCCTGCAA ACATCAGGCC CCCTCACCAC CACCGATAGC AGTACCCTTA CTATCACTGC    29940
CTCACCCCCT CTAACTACTG CCACTGGTAG CTTGGGCATT GACTTGAAAG AGCCCATTTA    30000
TACACAAAAT GGAAAACTAG GACTAAAGTA CGGGGCTCCT TTGCATGTAA CAGACGACCT    30060
AAACACTTTG ACCGTAGCAA CTGGTCCAGG TGTGACTATT AATAATACTT CCTTGCAAAC    30120
TAAAGTTACT GGAGCCTTGG GTTTTGATTC ACAAGGCAAT ATGCAACTTA ATGTAGCAGG    30180
AGGACTAAGG ATTGATTCTC AAAACAGACG CCTTATACTT GATGTTAGTT ATCCGTTTGA    30240
TGCTCAAAAC CAACTAAATC TAAGACTAGG ACAGGGCCCT CTTTTTATAA ACTCAGCCCA    30300
CAACTTGGAT ATTAACTACA ACAAAGGCCT TTACTTGTTT ACAGCTTCAA ACAATTCCAA    30360
AAAGCTTGAG GTTAACCTAA GCACTGCCAA GGGGTTGATG TTTGACGCTA CAGCCATAGC    30420
CATTAATGCA GGAGATGGGC TTGAATTTGG TTCACCTAAT GCACCAAACA CAAATCCCCT    30480
```

```
CAAAACAAAA ATTGGCCATG GCCTAGAATT TGATTCAAAC AAGGCTATGG TTCCTAAACT   30540

AGGAACTGGC CTTAGTTTTG ACAGCACAGG TGCCATTACA GTAGGAAACA AAAATAATGA   30600

TAAGCTAACT TTGTGGACCA CACCAGCTCC ATCTCCTAAC TGTAGACTAA ATGCAGAGAA   30660

AGATGCTAAA CTCACTTTGG TCTTAACAAA ATGTGGCAGT CAAATACTTG CTACAGTTTC   30720

AGTTTTGGCT GTTAAAGGCA GTTTGGCTCC AATATCTGGA ACAGTTCAAA GTGCTCATCT   30780

TATTATAAGA TTTGACGAAA ATGGAGTGCT ACTAAACAAT TCCTTCCTGG ACCCAGAATA   30840

TTGGAACTTT AGAAATGGAG ATCTTACTGA AGGCACAGCC TATACAAACG CTGTTGGATT   30900

TATGCCTAAC CTATCAGCTT ATCCAAAATC TCACGGTAAA ACTGCCAAAA GTAACATTGT   30960

CAGTCAAGTT TACTTAAACG GAGACAAAAC TAAACCTGTA ACACTAACCA TTACACTAAA   31020

CGGTACACAG GAAACAGGAG ACACAACTCC AAGTGCATAC TCTATGTCAT TTTCATGGGA   31080

CTGGTCTGGC CACAACTACA TTAATGAAAT ATTTGCCACA TCCTCTTACA CTTTTTCATA   31140

CATTGCCCAA GAATAAAGAA TCGTTTGTGT TATGTTTCAA CGTGTTTATT TTTCAATTGC   31200

AGAAAATTTC AAGTCATTTT TCATTCAGTA GTATAGCCCC ACCACCACAT AGCTTATACA   31260

GATCACCGTA CCTTAATCAA ACTCACAGAA CCCTAGTATT CAACCTGCCA CCTCCCTCCC   31320

AACACACAGA GTACACAGTC CTTTCTCCCC GGCTGGCCTT AAAAAGCATC ATATCATGGG   31380

TAACAGACAT ATTCTTAGGT GTTATATTCC ACACGGTTTC CTGTCGAGCC AAACGCTCAT   31440

CAGTGATATT AATAAACTCC CCGGGCAGCT CACTTAAGTT CATGTCGCTG TCCAGCTGCT   31500

GAGCCACAGG CTGCTGTCCA ACTTGCGGTT GCTTAACGGG CGGCGAAGGA GAAGTCCACG   31560

CCTACATGGG GGTAGAGTCA TAATCGTGCA TCAGGATAGG GCGGTGGTGC TGCAGCAGCG   31620

CGCGAATAAA CTGCTGCCGC CGCCGCTCCG TCCTGCAGGA ATACAACATG GCAGTGGTCT   31680

CCTCAGCGAT GATTCGCACC GCCCGCAGCA TAAGGCGCCT TGTCCTCCGG GCACAGCAGC   31740

GCACCCTGAT CTCACTTAAA TCAGCACAGT AACTGCAGCA CAGCACCACA ATATTGTTCA   31800

AAATCCCACA GTGCAAGGCG CTGTATCCAA AGCTCATGGC GGGGACCACA GAACCCACGT   31860

GGCCATCATA CCACAAGCGC AGGTAGATTA AGTGGCGACC CCTCATAAAC ACGCTGGACA   31920

TAAACATTAC CTCTTTTGGC ATGTTGTAAT TCACCACCTC CCGGTACCAT ATAAACCTCT   31980

GATTAAACAT GGCGCCATCC ACCACCATCC TAAACCAGCT GGCCAAAACC TGCCCGCCGG   32040

CTATACACTG CAGGGAACCG GGACTGGAAC AATGACAGTG GAGAGCCCAG GACTCGTAAC   32100

CATGGATCAT CATGCTCGTC ATGATATCAA TGTTGGCACA ACACAGGCAC ACGTGCATAC   32160

ACTTCCTCAG GATTACAAGC TCCTCCCGCG TTAGAACCAT ATCCCAGGGA ACAACCCATT   32220

CCTGAATCAG CGTAAATCCC ACACTGCAGG GAAGACCTCG CACGTAACTC ACGTTGTGCA   32280

TTGTCAAAGT GTTACATTCG GGCAGCAGCG GATGATCCTC CAGTATGGTA GCGCGGGTTT   32340

CTGTCTCAAA AGGAGGTAGA CGATCCCTAC TGTACGGAGT GCGCCGAGAC AACCGAGATC   32400

GTGTTGGTCG TAGTGTCATG CCAAATGGAA CGCCGGACGT AGTCATATTT CCTGAAGCAA   32460

AACCAGGTGC GGGCGTGACA AACAGATCTG CGTCTCCGGT CTCGCCGCTT AGATCGCTCT   32520

GTGTAGTAGT TGTAGTATAT CCACTCTCTC AAAGCATCCA GGCGCCCCCT GGCTTCGGGT   32580
```

●

```
TCTATGTAAA CTCCTTSAG CGCCGCTGCC CTGATAACAT CCACCACCGC AGAATAAGCC    32640

ACACCCAGCC AACCTACACA TTCGTTCTGC GAGTCACACA CGGGAGGAGC GGGAAGAGCT    32700

GGAAGAACCA TGTTTTTTTT TTTATTCCAA AAGATTATCC AAAACCTCAA AATGAAGATC    32760

TATTAAGTGA ACGCGCTCCC CTCCGGTGGC GTGGTCAAAC TCTACAGCCA AAGAACAGAT    32820

AATGGCATTT GTAAGATGTT GCACAATGGC TTCCAAAAGG CAAACGGCCC TCACGTCCAA    32880

GTGGACGTAA AGGCTAAACC CTTCAGGGTG AATCTCCTCT ATAAACATTC CAGCACCTTC    32940

AACCATGCCC AAATAATTCT CATCTCGCCA CCTTCTCAAT ATATCTCTAA GCAAATCCCG    33000

AATATTAAGT CCGGCCATTG TAAAAATCTG CTCCAGAGCG CCCTCCACCT TCAGCCTCAA    33060

GCAGCGAATC ATGATTGCAA AAATTCAGGT TCCTCACAGA CCTGTATAAG ATTCAAAAGC    33120

GGAACATTAA CAAAAATACC GCGATCCCGT AGGTCCCTTC GCAGGGCCAG CTGAACATAA    33180

TCGTGCAGGT CTGCACGGAC CAGCGCGGCC ACTTCCCCGC CAGGAACCTT GACAAAAGAA    33240

CCCACACTGA TTATGACACG CATACTCGGA GCTATGCTAA CCAGCGTAGC CCCGATGTAA    33300

GCTTTGTTGC ATGGGCGGCG ATATAAAATG CAAGGTGCTG CTCAAAAAAT CAGGCAAAGC    33360

CTCGCGCAAA AAAGAAAGCA CATCGTAGTC ATGCTCATGC AGATAAAGGC AGGTAAGCTC    33420

CGGAACCACC ACAGAAAAAG ACACCATTTT TCTCTCAAAC ATGTCTGCGG GTTTCTGCAT    33480

AAACACAAAA TAAAATAACA AAAAAACATT TAAACATTAG AAGCCTGTCT TACAACAGGA    33540

AAAACAACCC TTATAAGCAT AAGACGGACT ACGGCCATGC CGGCGTGACC GTAAAAAAAC    33600

TGGTCACCGT GATTAAAAAG CACCACCGAC AGCTCCTCGG TCATGTCCGG AGTCATAATG    33660

TAAGACTCGG TAAACACATC AGGTTGATTC ATCGGTCAGT GCTAAAAAGC GACCGAAATA    33720

GCCCGGGGGA ATACATACCC GCAGGCGTAG AGACAACATT ACAGCCCCCA TAGGAGGTAT    33780

AACAAAATTA ATAGGAGAGA AAAACACATA AACACCTGAA AAACCCTCCT GCCTAGGCAA    33840

AATAGCACCC TCCCGCTCCA GAACAACATA CAGCGCTTCA CAGCGGCAGC CTAACAGTCA    33900

GCCTTACCAG TAAAAAAGAA AACCTATTAA AAAAACACCA CTCGACACGG CACCAGCTCA    33960

ATCAGTCACA GTGTAAAAAA GGGCCAAGTG CAGAGCGAGT ATATATAGGA CTAAAAAATG    34020

ACGTAACGGT TAAAGTCCAC AAAAAACACC CAGAAAACCG CACGCGAACC TACGCCCAGA    34080

AACGAAAGCC AAAAAACCCA CAACTTCCTC AAATCGTCAC TTCCGTTTTC CCACGTTACG    34140

TAACTTCCCA TTTTAAGAAA ACTACAATTC CCAACACATA CAAGTTACTC CGCCCTAAAA    34200

CCTACGTCAC CCGCCCCGTT CCCACGCCCC GCGCCACGTC ACAAACTCCA·CCCCCTCATT    34260

ATCATATTGG CTTCAATCCA AAATAAGGTA TATTATTGAT GAT                       34303
```

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 380 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: other nucleic acid
        (A) DESCRIPTION: /desc = "DNA"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCAGGCTTTA CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA   60

ATTTCACACA GGAAACAGCT ATGACCATGA TTACGCCAAG CGCGCAATTA ACCCTCACTA   120

AAGGGAACAA AAGCTGGGTA CCGGGCCCCC CCTCGAGGTC GACGGTATCG ATAAGCTTAC   180

GCGTGGCCTA GGCGGCCGAA TTCCTGCAGC CCGGGGGATC CACTAGTTCT AGAGCGGCCG   240

CCACCGCGGC GCCTTAATTA ATACGTAAGC TCCAATTCGC CCTATAGTGA GTCGTATTAC   300

GCGCGCTCAC TGGCCGTCGT TTTACAACGT CGTGACTGGG AAAACCCTGG CGTTACCCAA   360

CTTAATCGCC TTGCAGCACA   380

(2) INFORMATION FOR SEQ ID NO:6:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 21 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: other nucleic acid
              (A) DESCRIPTION: /desc = "DNA"

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGCCGCAGCA CCGGATGCAT C   21

(2) INFORMATION FOR SEQ ID NO:7:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 27 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: other nucleic acid
              (A) DESCRIPTION: /desc = "DNA"

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGTCCGGAG GCTGCCATGC GGCAGGG   27

(2) INFORMATION FOR SEQ ID NO:8:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 1481 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: double
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: other nucleic acid
              (A) DESCRIPTION: /desc = "DNA"

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCTGCCATG CGGCAGGGAT ACGGCGCTAA CGATGCATCT CAACAATTGT TGTGTAGGTA   60

CTCCGCCGCC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC CTCTCGAGAA   120

AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC CGTGGCGGGC GGCAGCGGGC   180

GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC TGCTGATGAT GTAATTAAAG TAGGCGGTCT   240

TGAGACGGCG GATGGTCGAC AGAAGCACCA TGTCCTTGGG TCCGGCCTGC TGAATGCGCA   300

GGCGGTCGGC CATGCCCCAG GCTTCGTTTT GACATCGGCG CAGGTCTTTG TAGTAGTCTT   360

GCATGAGCCT TTCTACCGGC ACTTCTTCTT CTCCTTCCTC TTGTCCTGCA TCTCTTGCAT   420

- 104 -

CTATCGCTGC GGCGGCGGCG GAGTTTGGCC GTAGGTGGCG CCCTCTTCCT CCCATGCGTG   480

TGACCCCGAA GCCCCTCATC GGCTGAAGCA GGGCTAGGTC GGCGACAACG CGCTCGGCTA   540

ATATGGCCTG CTGCACCTGC GTGAGGGTAG ACTGGAAGTC ATCCATGTCC ACAAAGCGGT   600

GGTATGCGCC CGTGTTGATG GTGTAAGTGC AGTTGGCCAT AACGGACCAG TTAACGGTCT   660

GGTGACCCGG CTGCGAGAGC TCGGTGTACC TGAGACGCGA GTAAGCCCTC GAGTCAAATA   720

CGTAGTCGTT GCAAGTCCGC ACCAGGTACT GGTATCCCAC CAAAAGTGC GGCGGCGGCT   780

GGCGGTAGAG GGGCCAGCGT AGGGTGGCCG GGGCTCCGGG GGCGAGATCT TCCAACATAA   840

GGCGATGATA TCCGTAGATG TACCTGGACA TCCAGGTGAT GCCGGCGGCG GTGGTGGAGG   900

CGCGCGGAAA GTCGCGGACG CGGTTCCAGA TGTTGCGCAG CGGCAAAAAG TGCTCCATGG   960

TCGGGACGCT CTGGCCGGTC AGGCGCGCGC AATCGTTGAC GCTCTACCGT GCAAAAGGAG   1020

AGCCTGTAAG CGGGCACTCT TCCGTGGTCT GGTGGATAAA TTCGCAAGGG TATCATGGCG   1080

GACGACCGGG GTTCGAGCCC CGTATCCGGC CGTCCGCCGT GATCCATGCG GTTACCGCCC   1140

GCGTGTCGAA CCCAGGTGTG CGACGTCAGA CAACGGGGGA GTGCTCCTTT TGGCTTCCTT   1200

CCAGGCGCGG CGGCTGCTGC GCTAGCTTTT TTGGCCACTG GCCGCGCGCA GCGTAAGCGG   1260

TTAGGCTGGA AAGCGAAAGC ATTAAGTGGC TCGCTCCCTG TAGCCGGAGG GTTATTTTCC   1320

AAGGGTTGAG TCGCGGGACC CCCGGTTCGA GTCTCGGACC GGCCGGACTG CGGCGAACGG   1380

GGGTTTGCCT CCCCGTCATG CAAGACCCCG CTTGCAAATT CCTCCGGAAA CAGGGACGAG   1440

CCCCTTTTTT GCTTTTCCCA GATGCATCCG GTGCTGCGGC A   1481

(2) INFORMATION FOR SEQ ID NO:9:

          (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 3364 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: double
               (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: other nucleic acid
               (A) DESCRIPTION: /desc = "DNA"

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAATTCCCCA TCCTGGTCTA TAGAGAGAGT TCCAGAACAG CCAGGGCTAC AGATAAACCC   60

ATCTGGAAAA ACAAAGTTGA ATGACCCAAG AGGGGTTCTC AGAGGGTGGC GTGTGCTCCC   120

TGGCAAGCCT ATGACATGGC CGGGGCCTGC CTCTCTCTGC CTCTGACCCT CAGTGGCTCC   180

CATGAACTCC TTGCCCAATG GCATCTTTTT CCTGCGCTCC TTGGGTTATT CCAGTCTCCC   240

CTCAGCATTC CTTCCTCAGG GCCTCGCTCT TCTCTCTGCT CCCTCCTTGC ACAGCTGGCT   300

CTGTCCACCT CAGATGTCAC AGTGCTCTCT CAGAGGAGGA AGGCACCATG TACCCTCTGT   360

TTCCCAGGTA AGGGTTCAAT TTTTAAAAAT GGTTTTTTGT TTGTTTGTTT GTTTGTTTGT   420

TTGTTTGTTT TTCAAGACAG GGCTCCTCTG TGTAGTCCTA ACTGTCTTGA AACTCCCTCT   480

GTAGACCAGG TCGACCTCGA ACTCTTGAAA CCTGCCACGG ACCACCCAGT CAGGTATGGA   540

GGTCCCTGGA ATGAGCGTCC TCGAAGCTAG GTGGGTAAGG GTTCGGCGGT GACAAACAGA   600

AACAAACACA GAGGCAGTTT GAATCTGAGT GTATTTTGCA GCTCTCAAGC AGGGGATTTT   660

```
ATACATAAAA AAAAAAAACA AAAAAAAACC AAACATTACA TCTCTTAGAA ACTATATCCA    720

ATGAAACAAT CACAGATACC AACCAAAACC ATTGGGCAGA GTAAAGCACA AAAATCATCC    780

AAGCATTACA ACTCTGAAAC CATGTATTCA GTGAATCACA AACAGAACAG GTAACATCAT    840

TATTAATATA AATCACCAAA ATATAACAAT TCTAAAAGGA TGTATCCAGT GGGGGCTGTC    900

GTCCAAGGCT AGTGGCAGAT TTCCAGGAGC AGGTTAGTAA ATCTTAACCA CTGAACTAAC    960

TCTCCAGCCC CATGGTCAAT TATTATTTAG CATCTAGTGC CTAATTTTTT TTTATAAATC   1020

TTCACTATGT AATTTAAAAC TATTTTAATT CTTCCTAATT AAGGCTTTCT TTACCATATA   1080

CCAAAATTCA CCTCCAATGA CACACGCGTA GCCATATGAA ATTTTATTGT TGGGAAAATT   1140

TGTACCTATC ATAATAGTTT TGTAAATGAT TTAAAAAGCA AAGTGTTAGC CGGGCGTGGT   1200

GGCACACGCC TTTAATCCCT GCACTCGGGA GGCAGGGGCA GGAGGATTTC TGAGTTTGAG   1260

GCCAGCCTGG TCTACAGAGT GAGTTCCAGG ACAGCCAGGG CTACACAGAG AAACCCTGTC   1320

TCGAACCCCC CACCCCCCAA AAAAAGCAAA GTGTTGGTTT CCTTGGGGAT AAAGTCATGT   1380

TAGTGGCCCA TCTCTAGGCC CATCTCACCC ATTATTCTCG CTTAAGATCT TGGCCTAGGC   1440

TACCAGGAAC ATGTAAATAA GAAAAGGAAT AAGAGAAAAC AAAACAGAGA GATTGCCATG   1500

AGAACTACGG CTCAATATTT TTTCTCTCCG GCGAAGAGTT CCACAACCAT CTCCAGGAGG   1560

CCTCCACGTT TTGAGGTCAA TGGCCTCAGT CTGTGGAACT TGTCACACAG ATCTTACTGG   1620

AGGTGGTGTG GCAGAAACCC ATTCCTTTTA GTGTCTTGGG CTAAAAGTAA AAGGCCCAGA   1680

GGAGGCCTTT GCTCATCTGA CCATGCTGAC AAGGAACACG GGTGCCAGGA CAGAGGCTGG   1740

ACCCCAGGAA CACCTTAAAC ACTTCTTCCC TTCTCCGCCC CCTAGAGCAG GCTCCCCTCA   1800

CCAGCCTGGG CAGAAATGGG GGAAGATGGA GTGAAGCCAT ACTGGCTACT CCAGAATCAA   1860

CAGAGGGAGC CGGGGGCAAT ACTGGAGAAG CTGGTCTCCC CCCAGGGGCA ATCCTGGCAC   1920

CTCCCAGGCA GAAGAGGAAA CTTCCACAGT GCATCTCACT TCCATGAATC CCCTCCTCGG   1980

ACTCTGAGGT CCTTGGTCAC AGCTGAGGTG CAAAAGGCTC CTGTCATATT GTGTCCTGCT   2040

CTGGTCTGCC TTCCACAGCT TGGGGGCCAC CTAGCCCACC TCTCCCTAGG GATGAGAGCA   2100

GCCACTACGG GTCTAGGCTG CCCATGTAAG GAGGCAAGGC CTGGGGACAC CCGAGATGCC   2160

TGGTTATAAT TAACCCAGAC ATGTGGCTGC CCCCCCCCCC CCAACACCTG CTGCCTGAGC   2220

CTCACCCCCA CCCCGGTGCC TGGGTCTTAG GCTCTGTACA CCATGGAGGA GAAGCTCGCT   2280

CTAAAAATAA CCCTGTCCCT GGTGGATCCA GGGTGAGGGG CAGGCTGAGG GCGGCCACTT   2340

CCCTCAGCCG CAGGTTTGTT TTCCCAAGAA TGGTTTTTCT GCTTCTGTAG CTTTTCCTGT   2400

CAATTCTGCC ATGGTGGAGC AGCCTGCACT GGGCTTCTGG GAGAAACCAA ACCGGGTTCT   2460

AACCTTTCAG CTACAGTTAT TGCCTTTCCT GTAGATGGGC GACTACAGCC CCACCCCCAC   2520

CCCCGTCTCC TGTATCCTTC CTGGGCCTGG GGATCCTAGG CTTTCACTGG AAATTTCCCC   2580

CCAGGTGCTG TAGGCTAGAG TCACGGCTCC CAAGAACAGT GCTTGCCTGG CATGCATGGT   2640

TCTGAACCTC CAACTGCAAA AAATGACACA TACCTTGACC CTTGGAAGGC TGAGGCAGGG   2700

GGATTGCCAT GAGTGCAAAG CCAGACTGGG TGGCATAGTT AGACCCTGTC TCAAAAAACC   2760
```

●

AAAAACAATT AAATAACTAA AGTCAGGCAA GTAATCCTAC TCGGGAGACT GAGGCAGAGG   2820

GATTGTTACA TGTCTGAGGC CAGCCTGGAC TACATAGGGT TTCAGGCTAG CCCTGTCTAC   2880

AGAGTAAGGC CCTATTTCAA AAACACAAAC AAAATGGTTC TCCCAGCTGC TAATGCTCAC   2940

CAGGCAATGA AGCCTGGTGA GCATTAGCAA TGAAGGCAAT GAAGGAGGGT GCTGGCTACA   3000

ATCAAGGCTG TGGGGGACTG AGGGCAGGCT GTAACAGGCT TGGGGGCCAG GGCTTATACG   3060

TGCCTGGGAC TCCCAAAGTA TTACTGTTCC ATGTTCCCGG CGAAGGGCCA GCTGTCCCCC   3120

GCCAGCTAGA CTCAGCACTT AGTTTAGGAA CCAGTGAGCA AGTCAGCCCT TGGGGCAGCC   3180

CATACAAGGC CATGGGGCTG GGCAAGCTGC ACGCCTGGGT CCGGGGTGGG CACGGTGCCC   3240

GGGCAACGAG CTGAAAGCTC ATCTGCTCTC AGGGGCCCCT CCCTGGGGAC AGCCCCTCCT   3300

GGCTAGTCAC ACCCTGTAGG CTCCTCTATA TAACCCAGGG GCACAGGGGC TGCCCCCGGG   3360

TCAC
                                                                    3364

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 174 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "DNA"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGTACCACTA CGGGTCTAGG CTGCCCATGT AAGGAGGCAA GGCCTGGGGA CACCCGAGAT   60

GCCTGGTTAT AATTAACCCC AACACCTGCT GCCCCCCCCC CCCCAACACC TGCTGCCTGA   120

GCCTGAGCGG TTACCCCACC CCGGTGCCTG GGTCTTAGGC TCTGTACACC ATGG   174

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 699 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: other nucleic acid
            (A) DESCRIPTION: /desc = "DNA"

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGATCTTCCT AGAAATTCGC TGTCTGCGAG GGCCGGCTGT TGGGGTGAGT ACTCCCTCTC   60

AAAAGCGGGC ATGACTTCTG CGCTAAGATT GTCAGTTTCC AAAAACGAGG AGGATTTGAT   120

ATTCACCTGG CCCGCGGTGA TGCCTTTGAG GGTGGCCGCG TCCATCTGGT CAGAAAAGAC   180

AATCTTTTTG TTGTCAAGCT TGAGGTGTGG CAGGCTTGAG ATCGATCTGG CCATACACTT   240

GAGTGACAAT GACATCCACT TTGCCTTTCT CTCCACAGGT GTCCACTCCC AGGTCCAACC   300

GCGGATCTCC CGGGACCATG CCCAAGAAGA AGAGGAAGGT GTCCAATTTA CTGACCGTAC   360

ACCAAAATTT GCCTGCATTA CCGGTCGATG CAACGAGTGA TGAGGTTCGC AAGAACCTGA   420

TGGACATGTT CAGGGATCGC CAGGCGTTTT CTGAGCATAC CTGGAAAATG CTTCTGTCCG   480

```
TTTGCCGGTC GTGGGCGGCA TGGTGCAAGT TGAATAACCG GAAATGGTTT CCCGCAGAAC      540

CTGAAGATGT CGCGATTAT CTTCTATATC TTCAGGCGCG CGGTCTGGCA GTAAAAACTA      600

TCCAGCAACA TTTGGGCCAG CTAAACATGC TTCATCGTCG GTCCGGGCTG CCACGACCAA      660

GTGACAGCAA TGCTGTTTCA CTGGTTATGC GGCGGATCC                           699
```

(2) INFORMATION FOR SEQ ID NO:12:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 68 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE: other nucleic acid
           (A) DESCRIPTION: /desc = "DNA"

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
GAAGATCTAT AACTTCGTAT AATGTATGCT ATACGAAGTT ATTACCGAAG AAATGGCTCG      60

AGATCTTC                                                             68
```

(2) INFORMATION FOR SEQ ID NO:13:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 68 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: other nucleic acid
           (A) DESCRIPTION: /desc = "DNA"

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
GAAGATCTCG AGCCATTTCT TCGGTAATAA CTTCGTATAG CATACATTAT ACGAAGTTAT      60

AGATCTTC                                                             68
```

(2) INFORMATION FOR SEQ ID NO:14:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 50 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: other nucleic acid
           (A) DESCRIPTION: /desc = "DNA"

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
CCACATGTAT AACTTCGTAT AGCATACATT ATACGAAGTT ATACATGTGG              50
```

(2) INFORMATION FOR SEQ ID NO:15:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 50 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: other nucleic acid
           (A) DESCRIPTION: /desc = "DNA"

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
CCACATGTAT AACTTCGTAT AATGTATGCT ATACGAAGTT ATACATGTGG              50
```

CLAIMS

1.   A recombinant plasmid, comprising in operable combination:

a)   a plasmid backbone, comprising an origin of replication, an antibiotic resistance gene and a eukaryotic promoter element;

b)   the left and right inverted terminal repeats (ITRs) of adenovirus, said ITRs each having a 5' and a 3' end and arranged in a tail to tail orientation on said plasmid backbone;

c)   the adenovirus packaging sequence, said packaging sequence having a 5' and a 3' end and linked to one of said ITRs; and

d)   a first gene of interest operably linked to said promoter element.

2.   The recombinant plasmid of Claim 1 wherein the total size of said plasmid is between 27 and 40 kilobase pairs.

3.   The recombinant plasmid of Claim 1, wherein said 5' end of said packaging sequence is linked to said 3' end of said left ITR.

4.   The recombinant plasmid of Claim 3, wherein said first gene of interest is linked to said 3' end of said packaging sequence.

5.   The recombinant plasmid of Claim 4, wherein said first gene of interest is the dystrophin cDNA gene.

6.   The recombinant plasmid of Claim 4, further comprising a second gene of interest.

7.   The recombinant plasmid of Claim 6, wherein said second gene of interest is linked to said 3' end of said right ITR.

8.   The recombinant plasmid of Claim 7, wherein said second gene of interest is a reporter gene.

9. The recombinant plasmid of Claim 8. wherein said reporter gene is selected from the group consisting of the *E. coli* β-galactosidase gene. the human placental alkaline phosphatase gene. the green fluroescent protein gene and the choramphenicol acetyltransferase gene.

5   10. A mammalian cell line containing the recombinant plasmid of Claim 1.

11. The cell line of Claim 10. wherein said cell line is a 293-derived cell line.

12. A helper adenovirus comprising i) first and a second *loxP* sequences.
10   and ii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end.

13. The helper adenovirus of Claim 12. wherein said first *loxP* sequence is linked to the 5' end of said packaging sequence and said second *loxP* sequence is linked to said 3' end of said packaging sequence.

15   14. The helper adenovirus of Claim 12. further comprising at least one adenovirus gene coding region.

15. A mammalian cell line. comprising:

      a)   a recombinant plasmid. comprising. in operable combination:

           i)   a plasmid backbone. comprising an origin of
20   replication. an antibiotic resistance gene and a eukaryotic promoter element.

           ii)   the left and right inverted terminal repeats (ITRs) of adenovirus. said ITRs each having a 5' and a 3' end and arranged in a tail to tail orientation on said plasmid backbone.
25   iii)   the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end and linked to one of said ITRs. and

iv)   a first gene of interest operably linked to said promoter element: and

b)   a helper adenovirus comprising i) first and a second *loxP* sequences. and ii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end.

16.   The cell line of Claim 15. wherein said helper adenovirus further comprises at least one adenovirus gene coding region.

17.   The cell line of Claim 15. wherein said first gene of interest is the dystrophin cDNA gene

18.   A method of producing an adenovirus minichromosome. comprising:

A)   providing a mammalian cell line containing:

a)   a recombinant plasmid. comprising. in operable combination. i) a plasmid backbone. comprising an origin of replication. an antibiotic resistance gene and a eukarotic promoter element. ii) the left and right inverted terminal repeats (ITRs) of adenovirus. said ITRs each having a 5' and a 3' end and arranged in a tail to tail orientation on said plasmid backbone. iii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end and linked to one of said ITRs. and iv) a first gene of interest operably linked to said promoter element: and

b)   a helper adenovirus comprising i) first and a second *loxP* sequences. ii) at least one adenovirus gene coding region. and iii) the adenovirus packaging sequence. said packaging sequence having a 5' and a 3' end: and

B)   growing said cell line under conditions such that said adenovirus gene coding region is expressed and said recombinant plasmid directs the production of at least one adenoviral minichromosome.

19.   The method of Claim 18. wherein said adenovirus minichromosome is encapidated.

20. The method of Claim 18. further comprising 3) recovering said encapsidated adenovirus minichromosome.

21. The method of Claim 20. further comprising 4) purifying said recovered encapsidated adenovirus minichromosome.

22. The method of Claim 21. further comprisng 5) administering said purified encapsidated adenovirus minichromosome to a host.

23. The method of Claim 22. wherein said host is a mammal.

24. The method of Claim 23. wherein said mammal is a human.

25. A recombinant adenovirus comprising the adenovirus E2b region having a deletion. said adenovirus capable of self-propagation in a packaging cell line and said E2b region comprising the DNA polymerase gene and the adenovirus preterminal protein gene.

26. The recombinant adenovirus of Claim 25. wherein said deletion is within the adenovirus DNA polymerase gene.

27. The recombinant adenovirus of Claim 25. wherein said deletion is within the adenovirus preterminal protein gene.

28. The recombinant adenovirus of Claim 25. wherein said deletion is within the adenovirus DNA polymerase and preterminal protein genes.

29. A mammalian cell line stably and constitutively expressing the adenovirus E1 gene products and the adenovirus DNA polymerase.

30. The cell line of Claim 29. wherein said cell line comprises a recombinant adenovirus comprising a deletion within the E2b region. said recombinant adenovirus being capable of self-propagation in said cell line.

31.   The cell line of Claim 30. wherein said deletion within said E2b region comprises a deletion within the adenoviral DNA polymerase gene.

32.   The cell of Claim 29. wherein the genome of said cell line contains a nucleotide sequence encoding adenovirus DNA polymerase operably linked to a heterologous promoter.

33.   The cell line of Claim 32. wherein said cell line is selected from the group consisting of the B-6. B-9. C-1. C-4. C-7. C-13. and C-14 cell lines.

34.   The cell line of Claim 29 further constitutively expressing the adenovirus preterminal protein gene product.

35.   The cell line of Claim 34. wherein said cell line comprises a recombinant adenovirus comprising a deletion within the E2b region. said recombinant adenovirus being capable of self-propagation in said cell line.

36.   The cell line of Claim 35. wherein said deletion within said E2b region comprises a deletion within the adenoviral preterminal protein gene.

37.   The cell line of Claim 36. wherein said deletion within said E2b region comprises a deletion within the adenoviral DNA polymerase and preterminal protein genes.

38.   The cell line of Claim 34. wherein the genome of said cell line contains a nucleotide sequence encoding adenovirus preterminal protein operably linked to a heterologous promoter.

39.   The cell line of Claim 38. wherein said cell line is selected from the group consisting of the C-1. C-4. C-7. C-13. and C-14 cell lines.

- 113 -

40.   A method of producing infectious recombinant adenovirus particles containing an adenoviral genome containing a deletion within the E2b region, comprising

a)   providing:

          5   i)   a mammalian cell line stably and constitutively expressing the adenovirus E1 gene products and the adenovirus DNA polymerase;

                ii)   a recombinant adenovirus comprising a deletion within the E2b region, said recombinant adenovirus being capable of self-propagation in said cell line;

b)   introducing said recombinant adenovirus into said cell line under conditions such that said recombinant adenovirus is propagated to form infectious recombinant adenovirus particles; and

c)   recovering said infectious recombinant adenovirus particles.

41.   The method of Claim 40, further comprising d) purifying said recovered infectious recombinant adenovirus particles.

42.   The method of Claim 41, further comprising e) administering said purified recombinant adenovirus particles to a host.

43.   The method of Claim 40, wherein said mammalian cell line further constitutively expresses the adenovirus preterminal protein.

44.   A recombinant plasmid capable of replicating in a bacterial host comprising adenoviral E2b sequences, said E2b sequences containing a deletion within the polymerase gene, said deletion resulting in reduced polymerase activity.

45.   The recombinant plasmid of Claim 44, wherein said deletion comprises a deletion of nucleotides 8772 to 9385 in SEQ ID NO:4.

46.   The recombinant plasmid of Claim 45, wherein said plasmid has the designation pΔpol.

47.   The recombinant plasmid of Claim 45. wherein said plasmid has the designation pBHG11Δpol.

48.   A recombinant plasmid capable of replicating in a bacterial host comprising adenoviral E2b sequences. said E2b sequences containing a deletion within the preterminal protein gene. said deletion resulting in the inability to express functional preterminal protein without disruption of the VA RNA genes.

49.   The recombinant plasmid of Claim 48. wherein said deletion within said preterminal protein gene comprises a deletion of nucleotides 10.705 to 11.134 in SEQ ID NO:4.

50.   The recombinant plasmid of Claim 49. wherein said plasmid has the designation pΔpTP.

51.   The recombinant plasmid of Claim 49. wherein said plasmid has the designation pBHG11ΔpTP.

52.   The recombinant plasmid of Claim 48 further comprising a deletion within the polymerase gene. said deletion resulting in reduced polymerase activity.

53.   The recombinant plasmid of Claim 52. wherein said deletion within said polymerase and said preterminal protein genes comprises a deletion of nucleotides 8.773 to 9586 and 11.067 to 12.513 in SEQ ID NO:4.

54.   The recombinant plasmid of Claim 53. wherein said plasmid has the designation pAXBΔpolΔpTPVARNA+t13.

55.   The recombinant plasmid of Claim 53. wherein said plasmid has the designation pBHG11ΔpolΔpTPVARNA+t13.

FIGURE 1

A
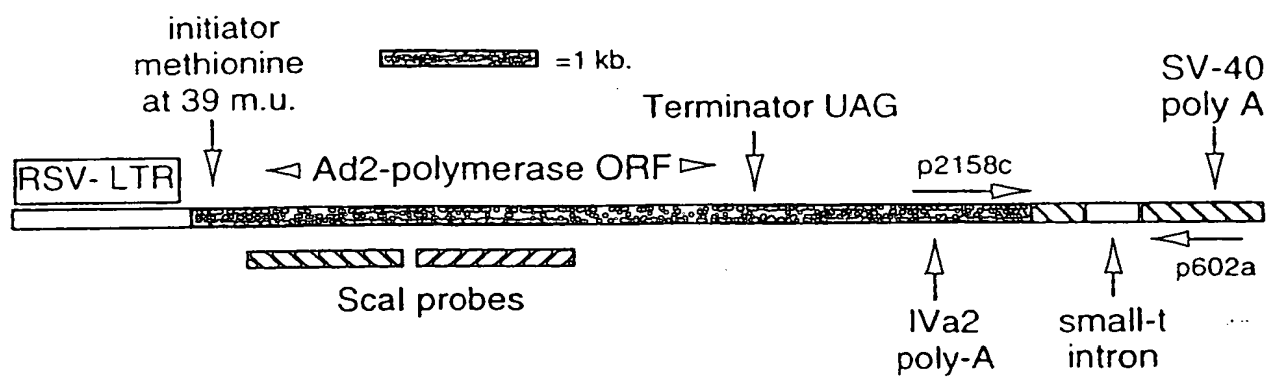
B
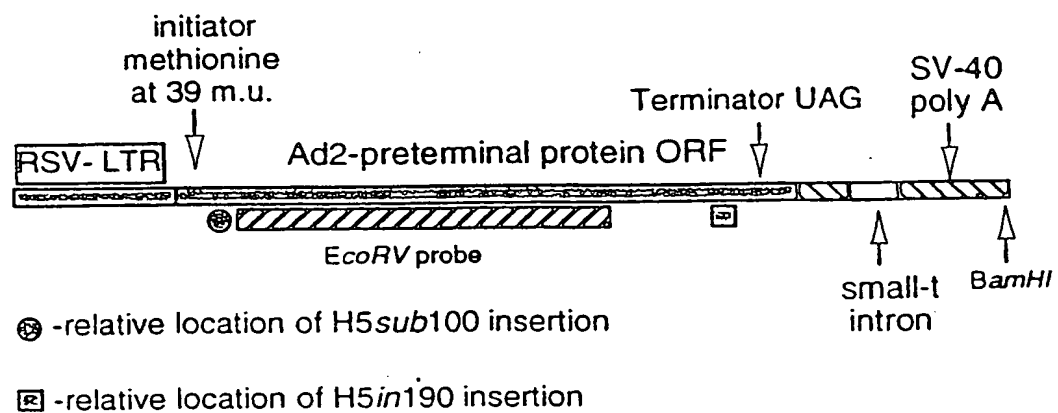
FIGURE 2
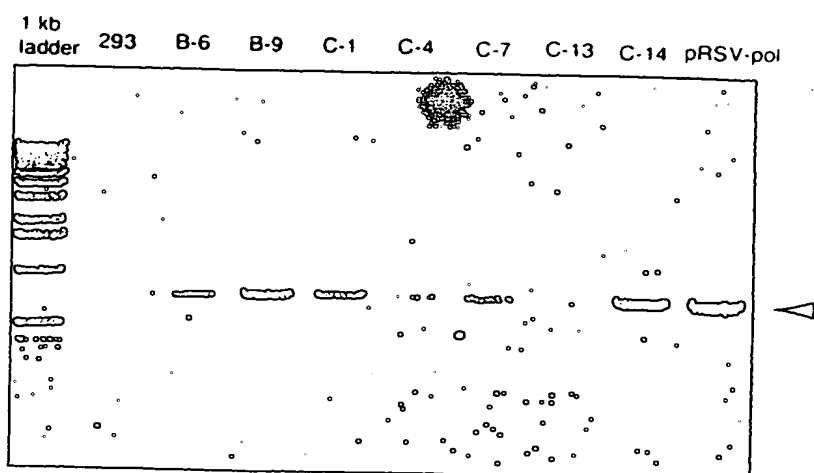
FIGURE 3

FIGURE 4

FIGURE 5

293 B-6 C-7
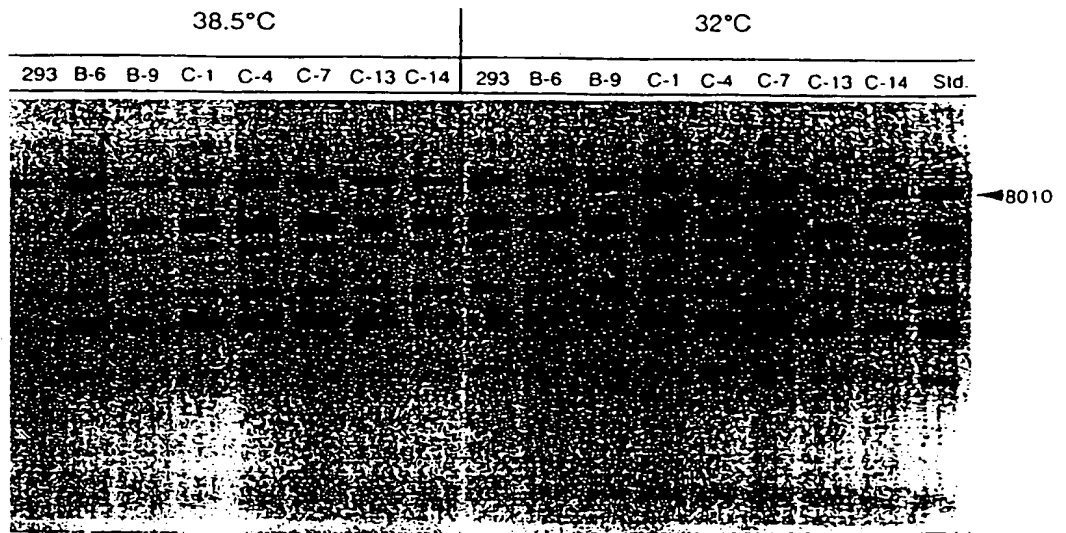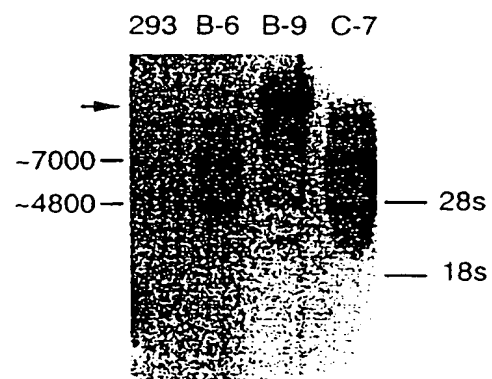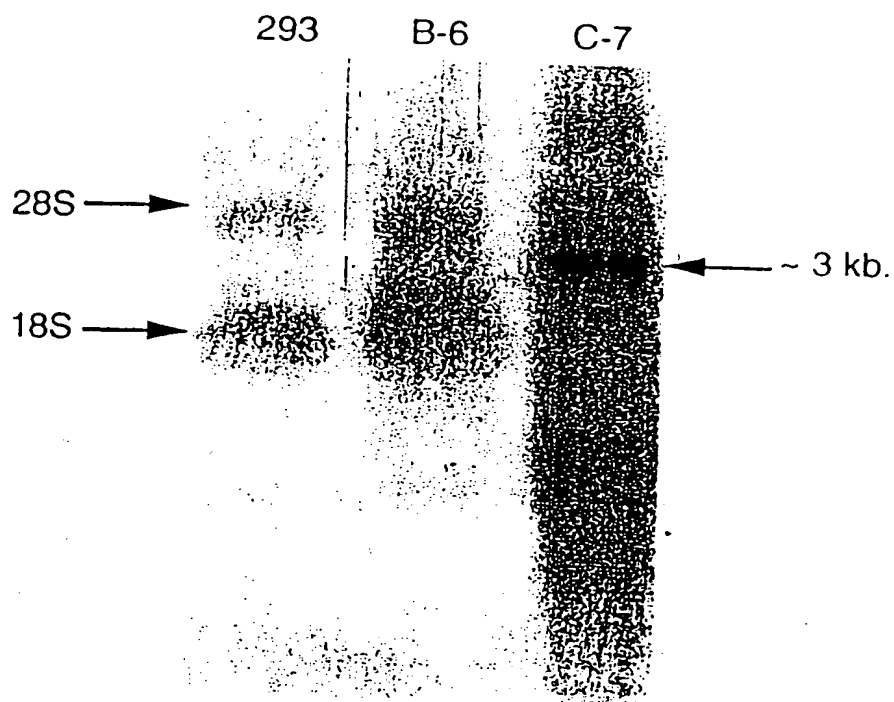
FIGURE 6

FIGURE 7

FIGURE 8

FIGURE 9

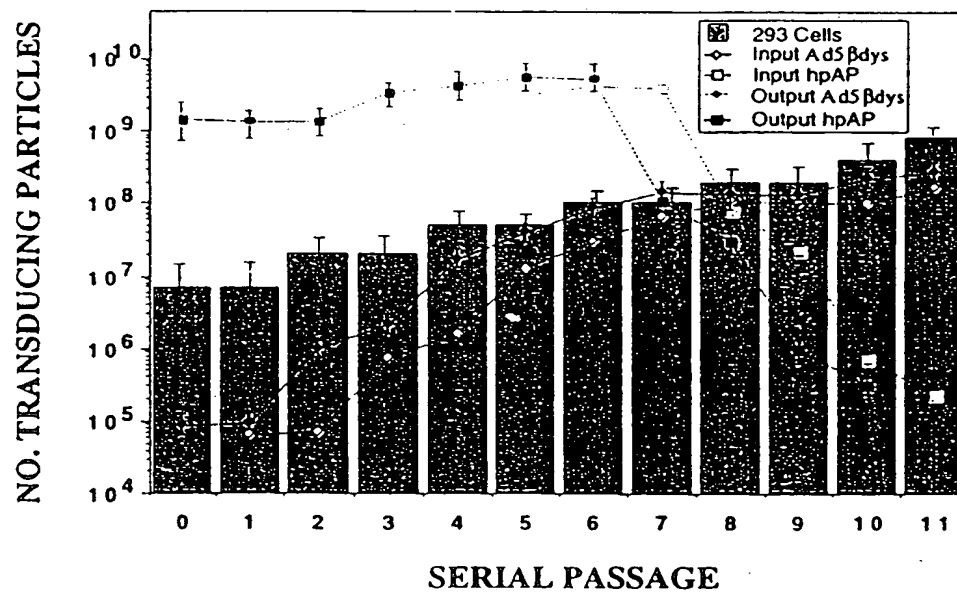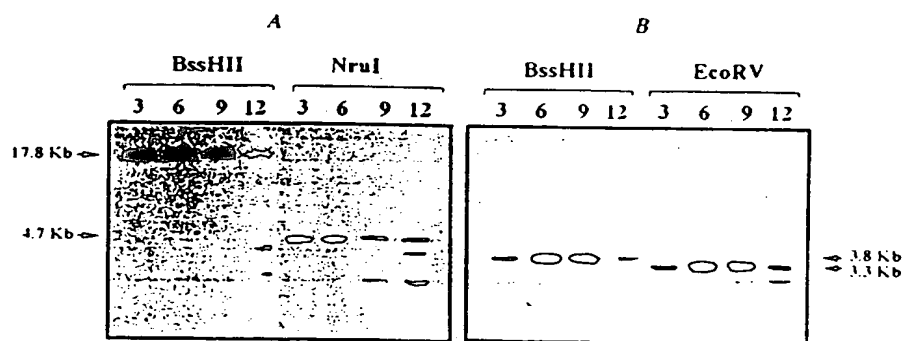
H5sub100 **Ad5ΔPOL**

32° 38.5° 37°
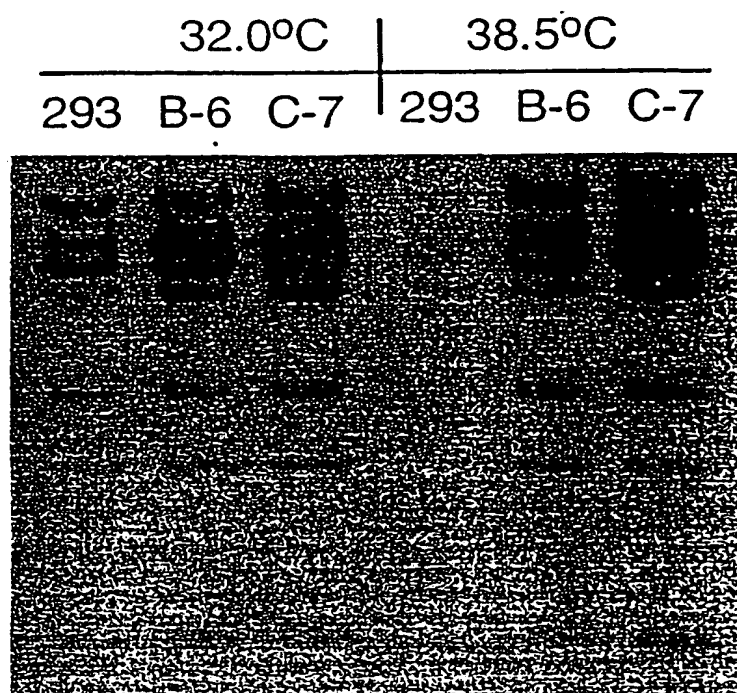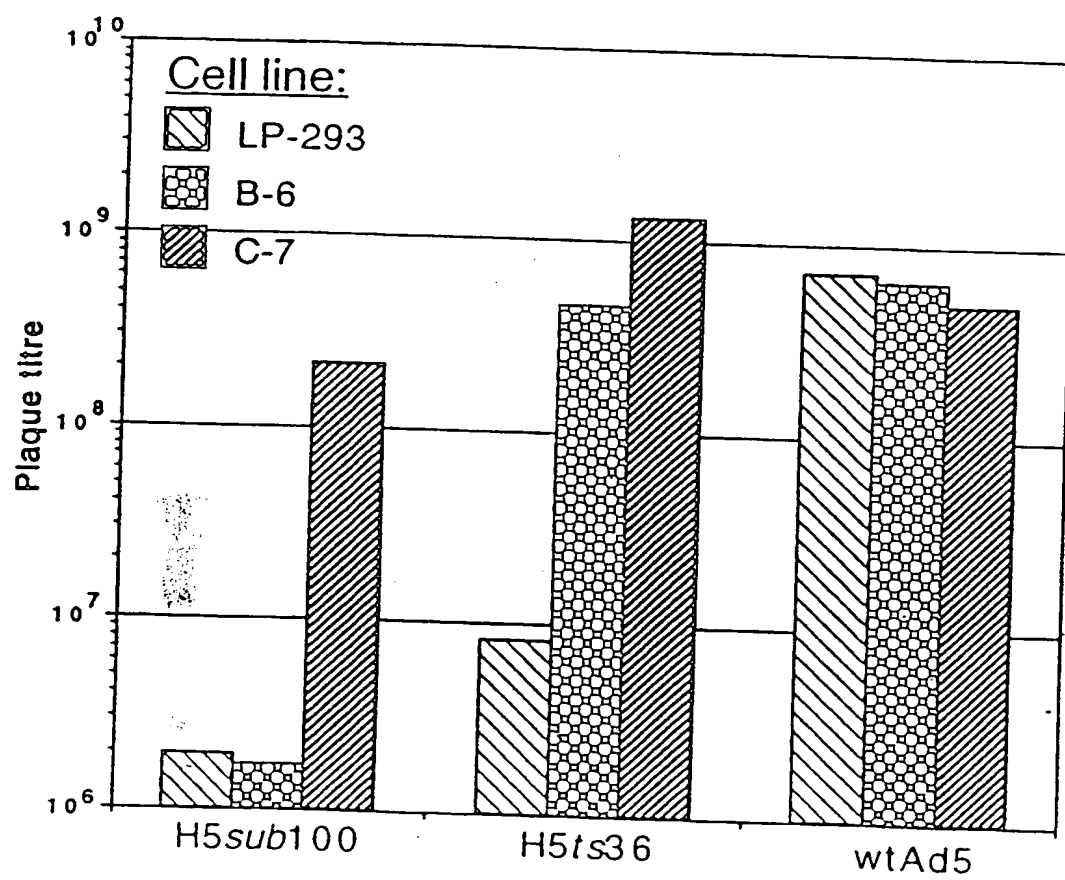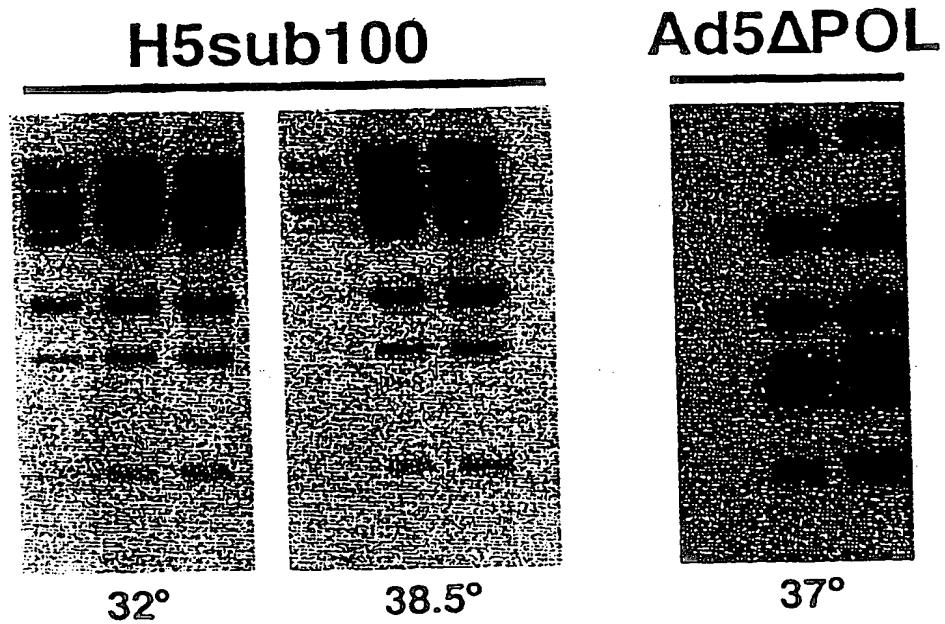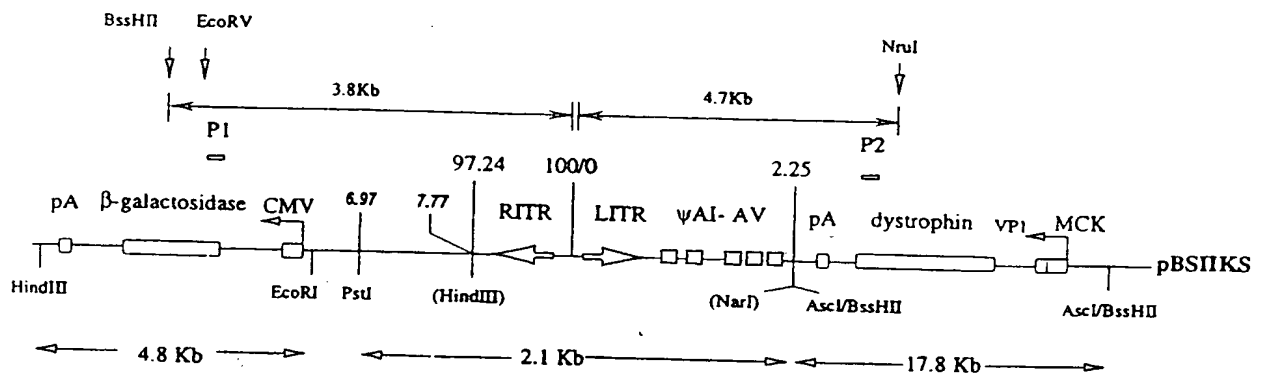
FIGURE 10

FIGURE 11

FIGURE 12

FIGURE 13
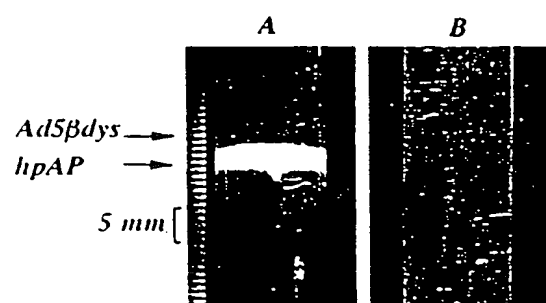
FIGURE 14

FIGURE 15

FIGURE 16

(A)   (B)   (C)
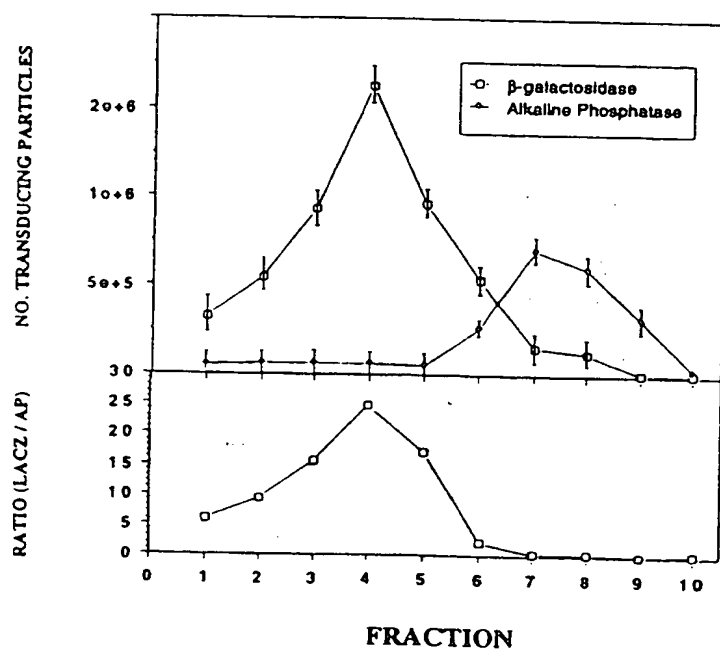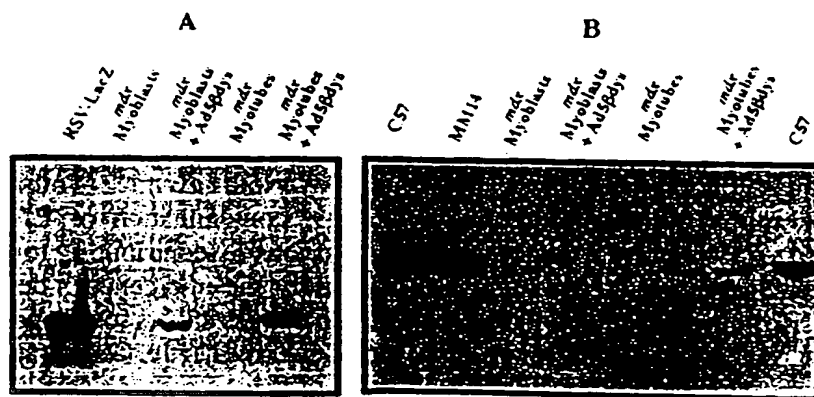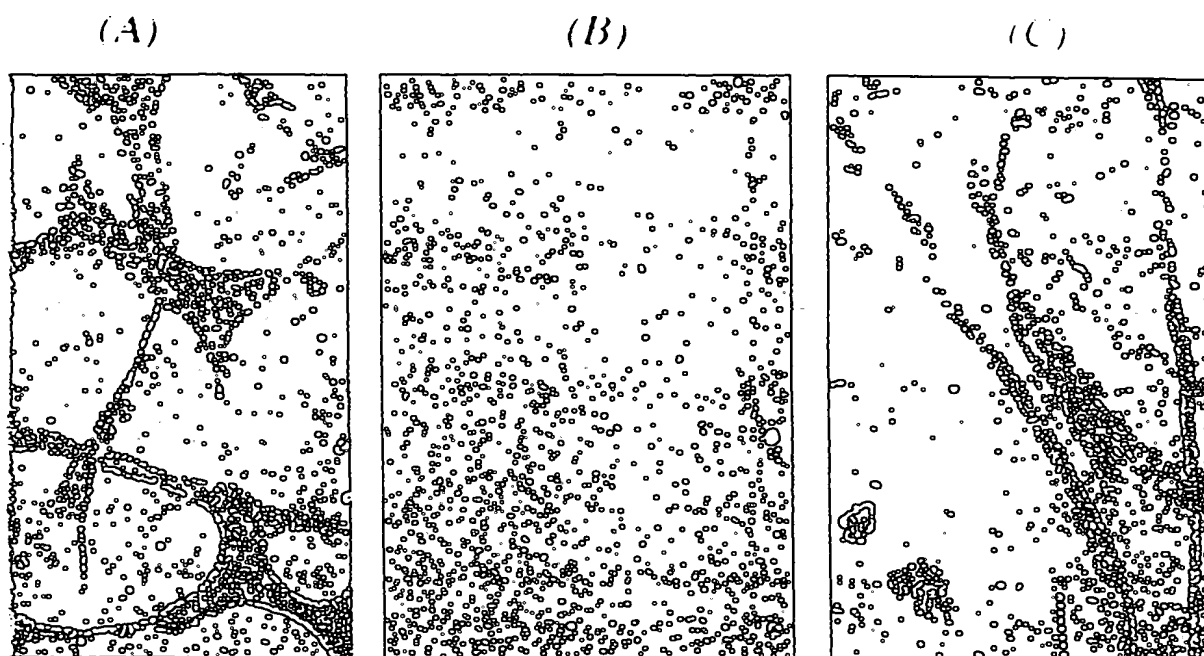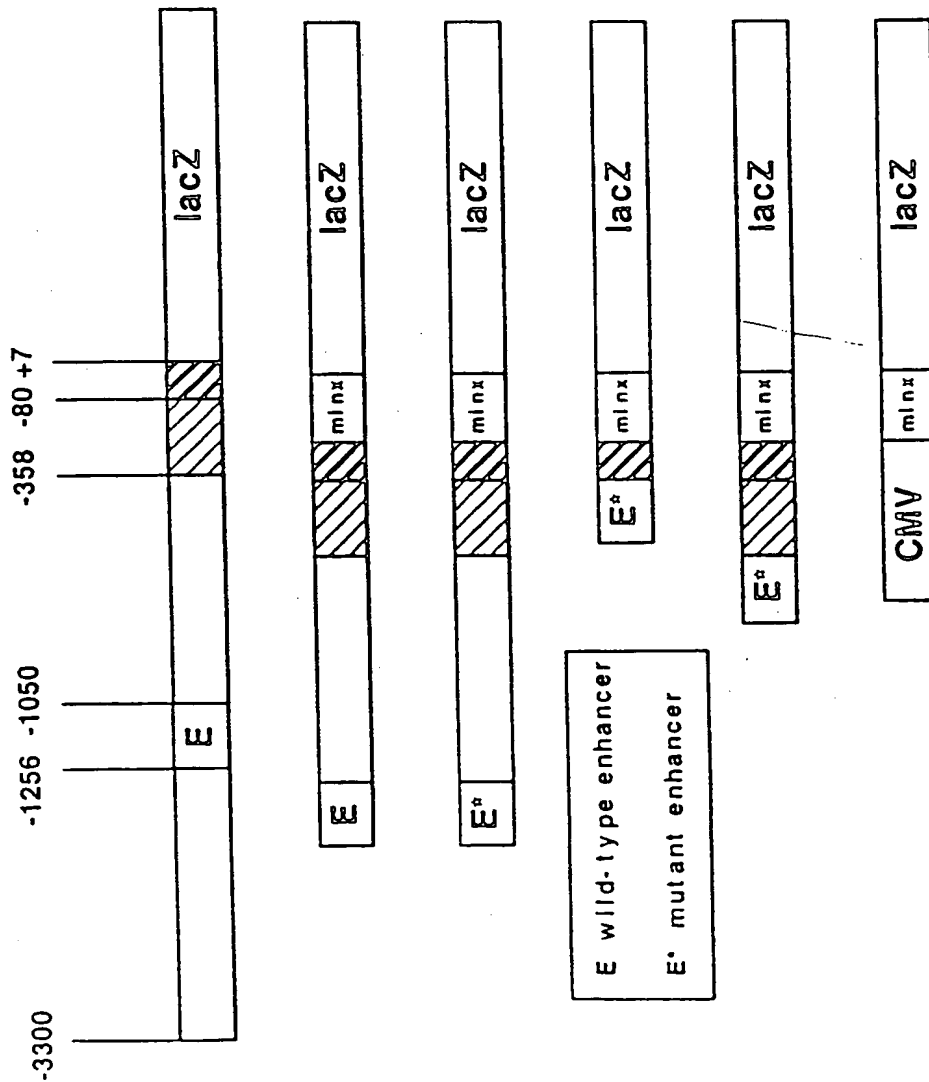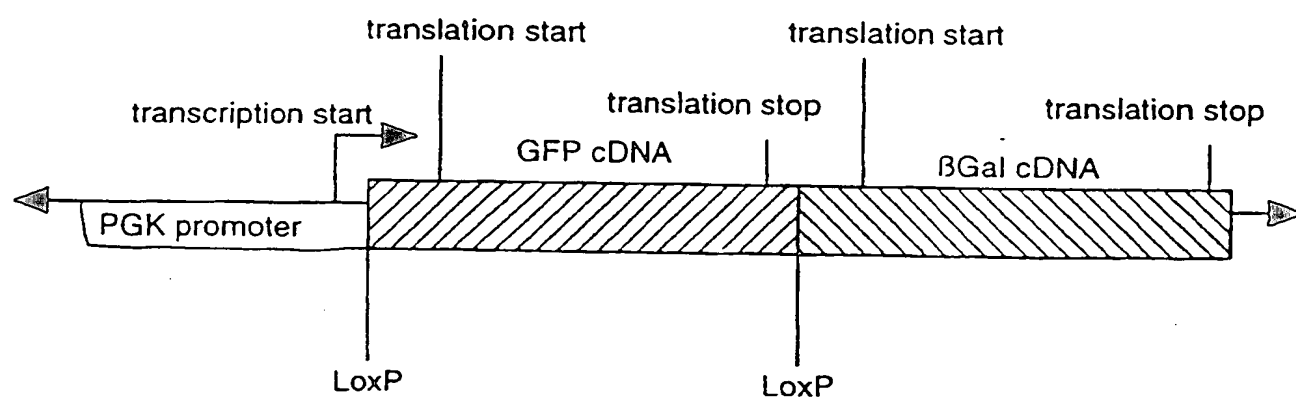
FIGURE 17

FIGURE 18

FIGURE 19
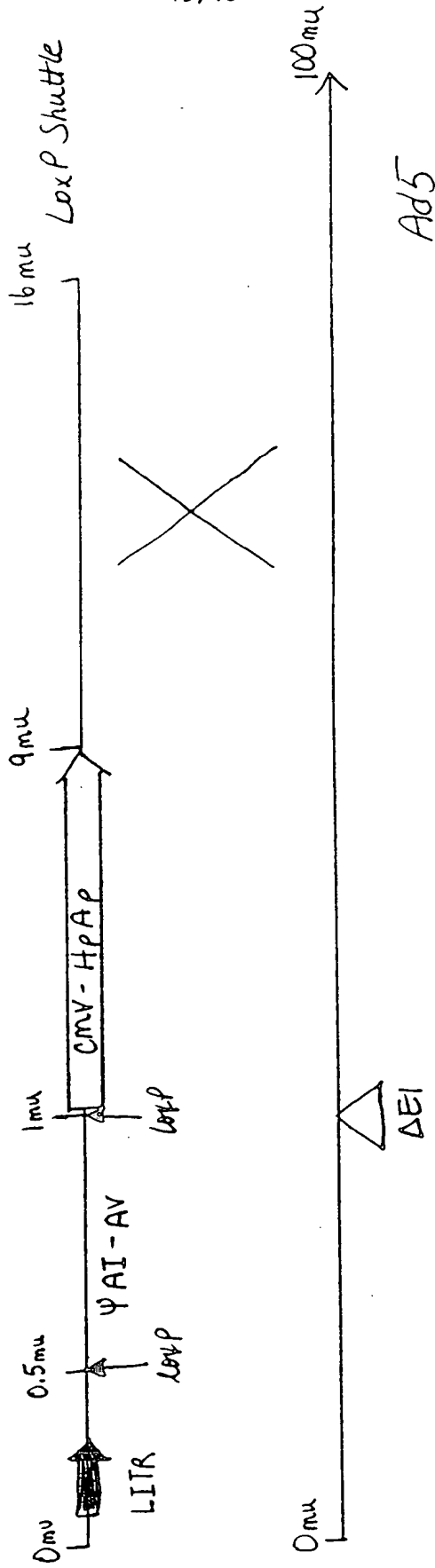
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/06, 5/10, 7/01, 7/02, 15/86

US CL :435/235.1, 320.1, 369

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1, 320.1, 369

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	KUMAR-SINGH et al. Encapsidated adenovirus minichromosomes allow delivery and expression of a 14 kb dystrophin cDNA to muscle cells. Hum. Mol. Genet. July 1996, Vol.5, No.7, pages 913-921, see entire document.	1-11 --- 15-24
X,P --- Y,P	PARKS et al. A helper-dependent adenovirus vector system: Removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc. Natl. Acad. Sci. USA. November 1996, Vol.93, pages 13565-13570, see entire document.	12-14 --- 15-24

X	Further documents are listed in the continuation of Box C.	☐	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 JANUARY 1998	10 FEB 1998

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ROBERT SCHWARTZMAN

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MITANI et al. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. Proc. Natl. Acad. Sci USA. April 1995, Vol.92, pages 3854-3858, especially pages 3855-3856.	25, 27
X	AMALFITANO et al. Improved adenovirus packaging cell lines to support the growth of replication-defective gene-delivery vectors. Proc. Natl. Acad. Sci. USA. April 1996, Vol.93, pages 3352-3356, see entire document.	29, 32, 33
X	CHAMBERLAIN et al. Packaging cells expressing the adenovirus (Ad) E1, polymerase, and preterminal proteins to allow the growth of a new class of replication defective Ad-vector for use in Duchenne muscular dystrophy (DMD). Am. J. Hum. Genet. 24 SEP 1996, Vol.59, No.4 Suppl., page A377, see entire document.	25-32, 34-38, 40-44, 48, 52
X	AMALFITANO et al. Modification of adenoviral vectors for use in gene therapy of Duchenne muscular dystrophy. Am. J. Hum. Genet. October 1995, Vol.57, No. 4 Suppl., page A234, see entire document.	25, 26, 29-33, 40, 41

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN: Medline, Biosis, Embase, CAPlus, WPIDS, JAPIO, PATOSEP, PATOSWO
APS
Search Terms: adenovirus, vector, LTR, inverted, E2B, DNA polymerase, preterminal protein, Cre, loxP